Leporipoxvirus Cu,Zn-Superoxide Dismutase (SOD) Homologs Are Catalytically Inert Decoy Proteins That Bind Copper Chaperone for SOD*  

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Melissa L. T. Teoh, Paula J. Walasek‡, and David H. Evans§  
From the Department of Molecular Biology & Genetics, University of Guelph, Guelph, Ontario N1G 2W1, Canada

Many Chordopoxviruses encode catalytically inactive homologs of cellular Cu-Zn superoxide dismutase (SOD). The biological function of these proteins is unknown, although the proteins encoded by Leporipoxviruses have been shown to promote a slow decline in the level of superoxide dismutase activity in virus-infected cells. To gain more insights into their function, we have further characterized the enzymatic and biochemical properties of a SOD homolog encoded by Shope fibroma virus. Shope fibroma virus SOD has retained the zinc binding properties of its cellular homolog, but cannot bind copper. Site-directed mutagenesis showed that it requires at least four amino acid substitutions to partially restore copper binding activity, but even these changes still did not restore catalytic activity. Reciprocal co-immunoprecipitation experiments showed that recombinant Shope fibroma virus SOD forms very stable complexes with cellular copper chaperones for SOD and these observations were confirmed using glutathione-S-transferase tagged proteins. Similar viral SOD/chaperone complexes were formed in cells infected with a closely related myxoma virus, where we also noted that some of the SOD antigen co-localizes with mitochondrial markers using confocal fluorescence microscopy. About 2% of the viral SOD was subsequently detected in gradient-purified mitochondria extracted from virus-infected cells. These poxviral SOD homologs do not form stable complexes with cellular Cu,Zn-SOD or affect its concentration. We suggest that Leporipoxivirus SOD homologs are catalytically inert decoy proteins that are designed to interfere in the proper mettallation and activation of cellular Cu,Zn-SOD. This reaction might be advantageous for tumorigenic poxviruses, since higher levels of superoxide have been proposed to have anti-apoptotic and tumorigenic activity.
these genes have clearly undergone an extensive process of mutagenesis, and this has had a particular effect of producing a catalytically inactive protein. What are the multiple mutations responsible for this genetic feature? The answer to this question would establish whether encoding a functional superoxide dismutase has, at any time in the recent evolutionary past, been of some selective advantage for these viruses. Second, it is unclear from a comparison of the protein sequences of viral and cellular Cu,Zn-SOD homologs, what effect the many amino acid substitutions and small deletions have had on the metal binding properties of these proteins. Do the viral enzymes retain the capacity to bind metals and thus perhaps function as Zn and/or Cu chelators? Finally, poxviral Cu,Zn-SODs conserve many of the amino acids that form the interface between cellular Cu,Zn-SOD homodimers and between Cu,Zn-SOD and CCS heterodimers (Fig. 1). If these poxviral proteins have retained the capacity to associate with cellular Cu,Zn-SOD and/or CCS, this would provide a possible mechanism by which leporipoxviruses could perturb the level of cellular superoxide dismutase activity.

In the studies that follow we show that Leporipoxvirus SOD homologs have lost the capacity to bind copper but retain the capacity to bind the copper chaperone and bind zinc. These biochemical properties suggest a novel way in which myxoma and Shope fibroma viruses might be using Cu,Zn-SOD homologs to manipulate intracellular concentrations of O$_2^•$ and thus promote the growth of virus-infected cells.

EXPERIMENTAL PROCEDURES

Virus and Virus Culture—All cell lines (BSC40, BGMK, and SIRC) were cultured in Dulbecco’s modified Eagle’s media supplemented with 10% fetal bovine serum (16). Myxoma virus (strain Laussanne) and vaccinia virus (strain WR) were obtained originally from the American type culture collection, and a vaccinia strain encoding T7 RNA polymerase (VTF7.5) was obtained from Dr. P. Traktman (University of Wisconsin). The construction of a recombinant myxoma virus encoding a deletion of MYX Cu,Zn-SOD (myxM131R) and a vaccinia virus encoding SFV Cu,Zn-SOD under the regulation of a T7 promoter (VMM5.2) is described elsewhere (16). Viruses were routinely passaged on BGMK or SIRC cells. Expression of recombinant SFV Cu,Zn-SOD in mammalian cells was accomplished by co-infecting BSC40 or BGMK cells with vaccinia VMM5.2 plus VTF7.5 at a multiplicity of two of each virus (16).

Plasmids and Site-directed Mutagenesis—Plasmid pDE422 (18) encodes the SFV Cu,Zn-SOD open-reading frame (S131R) fused in-frame with glutathione S-transferase (GST) in pGE2X (Amerham Biosciences). A standard PCR-based mutagenesis technique and mutagenic PCR primers were used to sequentially introduce amino acid substitutions into the SOD domain of the fusion protein. Table I illustrates the primers used and the resulting amino acid substitutions. Multiple amino acid substitutions were introduced in the order H148T→N59H→W157C→V157R creating mutant proteins designated S131R-T, S131R-TH, S131R-THC, and S131R-THCR, respectively. The W157C substitution mutation was also introduced separately into the cloned SFV S131R gene, creating a protein designated S131R-C. All plasmids were sequenced to confirm the presence of the introduced substitution mutations and to ensure that no additional mutations were present. A plasmid encoding a protein comprising GST fused to full-length human copper chaperone for SOD (CCS) in pGE2X was obtained from Dr. J. Gitlin (Washington University School of Medicine) (20). Protein expression studies used Escherichia coli strain BL21.

Purification of GST-tagged Proteins—GST-tagged proteins were prepared following a modified commercial protocol (Amersham Biosciences). Bacterial cultures were grown to an optical density of one at 30 °C in the presence of 50 μg/ml ampicillin and induced with 0.2 mM isopropyl-1-thio-β-D-galactopyranoside. CuSO$_4$ and ZnSO$_4$ were added to final concentrations of 0.1 mM, 30 min later, and the cells were harvested by centrifugation 2 h post-induction. The cell pellet was washed with ice-cold lysis buffer and disrupted with a Dounce homogenizer in PBS containing 1 mM phenylmethylsulfonyl fluoride, 10 mM EDTA, 75 μg/ml lysozyme, 5 mM dithi-othreitol (DTT), and 1% Triton X-100. The debris was removed by centrifugation, and GST-tagged proteins were recovered by chromatography using a GSTrap column. Proteins were eluted with 10 mM reduced glutathione in 50 mM Tris-HCl (pH 8.0). Where indicated, GST-tagged proteins were cleaved overnight at room temperature in Tris-HCl with one cleavage unit of thrombin (Sigma) per 100 μg of fusion protein.

Metal Analysis—Affinity-purified GST fusion proteins were dialedyzed extensively against 2000 volumes of Chelex-100 treated 25 mM phosphate buffer (pH 7.8) at 4 °C. Protein concentration was determined using a dye-binding assay (Bio-Rad) and a bovine serum albumin protein standard, and the copper and zinc contents were determined using a Varian 3000 graphite furnace atomic absorption spectrometer. Dialyzed bovine Cu,Zn-SOD (Sigma) was used as a control and reference for metal content. The metal content of GST was also measured, and we determined <0.2 μg of zinc and no detectable copper per mole of GST protein.

Cu,Zn-SOD Assays—Superoxide dismutase activity was measured by monitoring the inhibition of reduction of ferricytochrome c by hypoxanthine/xanthine oxidase (21). A solution, containing the test sample and 12 milliliters/ml xanthine oxidase in 0.25 ml of PBS, was added to 0.25 ml of PBS containing 120 units/ml catalase, 0.2 mM hypoxanthine, and 0.1 mM ferricytochrome c. The sample was incubated for 5 min at room temperature, and ΔA$_{550}$ was monitored over an additional 2 min. One unit of activity is the amount of protein required to inhibit the reduction of cytochrome c by 50%.

Protein Binding Assays—GST “pull-down” assays used extracts prepared from bacteria expressing a GST-CCS fusion protein. About 50 μl of 50% v/v glutathione-Sepharose beads (Sigma) was added to 1.0 ml of clarified bacterial lysate containing ~80 μg of fusion protein and incubated at 4 °C for 1 h. The beads were recovered by centrifugation, washed three times with PBS, and then incubated with ~0.9 mg of mammalian cell protein in 0.2 ml of PBS at 4 °C for 2 h. The beads were washed extensively with PBS, and the protein complexes were released by adding 0.1 ml of SDS-PAGE loading buffer containing 0.1 M DTT, plus 2% β-mercaptoethanol, and heating at 100 °C for 5 min. Human Cu,Zn-SOD was used as a positive control (Sigma).

Immunoprecipitation assays used extracts isolated from virus-infected BGMK cells. The cells were harvested 15–24 h post-infection by lysis with a buffer containing 1% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, 1 μM aprotinin, and 1 μg/ml leupeptin, and the insoluble material was removed by centrifugation. The supernatant (~600 μl) was incubated overnight at 4 °C with 1.50 diluted preimmune rabbit serum and ~50 μl (packed volume) of protein A beads (Sigma) and pre-cleared by centrifugation. The protein content of the supernatant was determined, and 1 mg of protein extract (~600 μl) was mixed with 5 μg of rabbit anti-CCS or murine antiSFV SOD antibody and incubated at 4 °C for 2 h. Protein A beads were used to retrieve the complexes that were then washed three times with Nonidet P-40 lysis buffer. Proteins were released from the beads by boiling them for 5 min in 0.2 ml of SDS-PAGE loading buffer containing 0.1 M DTT. These interaction assays were repeated three times with similar results.

SDS-PAGE and Western Blot Analysis—Proteins were size-fractionated using 12% SDS-PAGE (22) and transferred to a polyvinylidene difluoride support as directed by the manufacturer of the membrane (Millipore). The membrane was treated with 5% blocking agent (Amer sham Biosciences) in PBST (PBS containing 0.1% Tween 20) and then incubated with either monoclonal aSFV SOD antibody (diluted 1:1000) or polyclonal aCCS antibodies (1:5000) in PBST for 1 h at room temperature. The blot was washed six times with PBST and incubated with 1:50,000 diluted secondary antibody conjugated to horseradish peroxidase as directed by the manufacturer of the membrane (Millipore). The membrane was blocked with 5% blocking agent (Amer sham Biosciences) in PBST (PBS containing 0.1% Tween 20) and then incubated with either monoclonal aSFV SOD antibody (diluted 1:1000) or polyclonal aCCS antibodies (1:5000) in PBST for 1 h at room temperature. The membranes were washed extensively, and horseradish peroxidase was detected using a chemiluminescent detection reagent (Pierce) and Kodak X-OMAT film. The monoclonal mouse aSFV SOD antibody detects both MYX and SFV SOD homologs but not cellular Cu,Zn-SOD (16). The polyclonal rabbit aCCS antibodies were kindly provided by Dr. J. Gitlin (20). They target human CCS domains I and III (9) and thus do not cross-react with cellular Cu,Zn-SOD. A polyclonal sheep a-human Cu,Zn-SOD antibody was generated from bioderived fusion protein used for Western blotting (data not shown).

Confocal Fluorescence Microscopy—A Leica TCS SP2 confocal microscope was used to investigate the distribution of MYX SOD antigen. BGMK cells were seeded on glass coverslips, infected (or mock infected) with myxoma virus at a multiplicity of infection of 20, and cultured 24 h post-infection. The day after, the cells were washed, fixed for 15 min with 3% formaldehyde in fresh pre-warmed media, washed with PBS, and permeabilized during a
Structural Features of SFV and Other Poxvirus Cu,Zn-SOD Homologs—Figure 1 shows a comparison of the amino acid sequences of the SOD-like proteins encoded by poxviruses with the homologous sequences of bovine Cu,Zn-SOD and the SOD-like domain II of human CCS (hCCS). This multiple sequence alignment illustrates the multiple deletions in the gene that seem to have occurred during the evolution of orthopoxviruses like ectromelia, vaccinia, and variola. These deletions would be expected to delete all the residues shown from crystallographic analysis of bovine Cu,Zn-SOD (24) to form the zinc binding domain (Fig. 1, filled circles) and results in this family of virus genes segregating to a different branch of a SOD-based “relatedness” tree (Fig. 1, lower panel). In contrast the SFV S131R gene product, and similar “non-orthopoxvirus” proteins, seem to have retained many of the structural features of Cu,Zn-SODs, including the amino acids that form the interface between SOD homodimers (Fig. 1, asterisks). SFV SOD has also retained homologs of the amino acids that in the yeast CCS/SOD heterodimeric structure (11) form key interactions between CCS and Cu,Zn-SOD (Fig. 1, small open circles). The residues responsible for binding zinc have also been conserved in SFV SOD, but an H → N substitution would delete at least one of the copper-coordinating residues (Fig. 1, large open circles). The capacity to form an intramolecular cross-link also seems to have been impaired by a C → W substitution and possibly by the displacement of the cysteine partner, and an R → V substitution is expected to delete an arginine involved in catalysis (24). Similar sequence changes characterize the gene encoded by myxoma virus and to viruses belonging to the Capripoxvirus family, whereas the protein encoded by Amsacta morei entomopoxivirus is sufficiently similar to cellular Cu,Zn-SOD orthologs to account for its being catalytically active.2

Interestingly, Lamb et al. (11) noted that forming a SOD-CCS heterodimer leads to an exchange of disulfide bonds. An intras- trand bond between Cys-57 and Cys-146, seen in the yeast SOD homodimer, is replaced in the SOD-CCS complex by an inter- strand disulfide bond between the SOD monomer and domain III of yeast CCS (SOD Cys-57 and CCS Cys-229). Molecular modeling suggests that amino acid Cys-67 in the Leporipoxivi- rus SOD homologs occupies a position very similar to that of Cys-57 in yeast SOD, within loop 4 and corresponding to the S-S subloop region of the cellular enzymes. Viral SOD homologs don’t encode a second homologous cysteine that would be well positioned to form an intrastrand disulfide bond with viral residue Cys-67. Instead, this residue would be predicted to protrude outwards and facing the surface of the protein where it would be well positioned to form an intermolecular disulfide bond with CCS Cys-229.

Site-directed Mutagenesis and Purification of Recombinant SFV S131R Protein—To gain some insights into the impact of these different amino acid substitutions upon the enzymatic and metal-binding properties of SFV SOD, we introduced a series of amino acid substitution mutations into the SFV S131R gene, in an attempt to return the sequence to something more closely resembling a catalytically active superoxide dismutase. The sequence alterations are shown in Table I. The W157C mutation (numbering refers to the SFV S131R gene) was introduced to restore some potential for forming an intrastrand disulfide bond with the semi-conserved cysteine at S131R position 67. Based upon molecular modeling of the structure of the copper binding cleft in SFV SOD, the two H148T and N59H substitutions were expected to restore the original copper-coordinating domain, and the V154R mutation was introduced to restore a highly conserved arginine that comprises part of the bovine Cu,Zn-SOD catalytic site. These S131R mutant proteins, along with the wild-type protein, were expressed in E. coli as GST fusion proteins, purified using pGEX-4T-1 based spectrophotometric method, and for the most part caused no obvious toxicity. The growth of bacterial strains encoding the quadruple mutant S131R-THCR was slowed significantly upon inducing the expression of this particular polypeptide, but this effect was overcome by adding CuSO4 and ZnSO4 to the media.

Multiple Mutations Prevent SFV SOD From Behaving as a Superoxide Dismutase—Despite introducing up to four poten- tially restorative amino acid substitution mutations into SFV SOD, we were unable to restore any significant additional dismutase activity to the protein. Our previous work had used a semi-quantitative in situ gel staining method to show that the native protein exhibited less than 1% of the activity of bovine or human Cu,Zn-SOD (16). Using a more quantitative cytochrome c-based spectrophotometric method, we observed that the wild-type protein retains almost exactly 1% of the activity of the purified bovine enzyme (Fig. 3). No significant changes in this level of activity were obtained with the introduction of any of the amino acid substitutions, even in the quadruple S131R-THCR mutant protein. It also made no dif- ference to the level of activity whether, or not, SFV SOD was cleaved from the GST fusion protein (Fig. 3), and incubating

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2. R. Moyer and M. Becker, personal communication.
MONTHLY SEQUENCE ALIGNMENT OF CELLULAR AND VIRAL Cu,Zn-SOD HOMOLOGS.

ClustalW (33) was used to align the indicated protein sequences and this alignment was used to generate an average distance tree. Orthopoxvirus SOD homologs are encoded by ectromelia (EYM-144), cowpox (CPXV-187), camelpox (CMLV-165), variola (VAR_Bang-A49R), vaccinia strain MVA (VV_MVA-158R), rabbitpox (RPV-157), vaccinia strain Copenhagen (VV_Cop-A45R), and monkeypox (MPV-Zar-A46R) viruses. Capripoxviruses include sheeppox (ShPV_TU-126), lumpy skin disease virus (LSDV-131), and swinepox (SPV-129) viruses.\footnote{Lesopoxviruses are myxoma (MYX-m131R) and Shope fibroma (SFV-s131R) viruses.} The alignment also includes an Amsacta morei entomopoxvirus gene (AmEPV-255), bovine Cu,Zn-SOD (Bovin SOD, Bovin), and residues 71–235 from domain II of human CCS (CCS_HUMAN). The figure also shows residues found from x-ray structures to form the SOD-SOD and SOD/\textit{H18528}\textit{CCS} interface (11). The positions of the four amino acid substitutions that were introduced into SFV SOD in an attempt to restore catalytic activity are also noted (N59H, H148T, V154R, and W157C).
Mutations were introduced in the order H148T → N59H → V157C → V157R as well as W157C separately. The quadruple mutant S131R-THCR might be expected to restore the copper-coordinating domain (H148T + V154R), an intras Strand disulfide bond (W157C), and a catalytic arginine (V154R).

Table 1

| Mutant | Oligonucleotides (5’ → 3’) | Amino acid substitution(s) |
|--------|--------------------------|---------------------------|
| S131R-C | AGTTATCAGCCTGTGGGATTATAAGGTTTGGCT ATCCCAAGGGGATAACGTACACGGAATTACC | W157C |
| S131R-T | AGTTCCGATAACCCGTAATTCCCGTTAAGTT GAATTTACGGTTATACGAACTGTTCACTGTT | H148T |
| S131R-TH | CACGTTATACGCTTCAATGGTTTTGAGAT TCATGAAACTGTATACCGGTTAATGCTTCACT | H148T + N59H |
| S131R-THC | AGTTATCAGCCTGTGGGATTATAAGGTTTGGCT ATCCCAAGGGGATAACGTACACGGAATTACC | H148T + N59H + W157C |
| S131R-THCR | AGGCCATGGCGGTTTCCCGAATTACCGGTTAT GGAACCGCATCGCCTGTGGGATTATAGGAGTTGCT | H148T + N59H + W157C + V154R |

Fig. 2. SDS-PAGE analysis of recombinant forms of SFV S131R protein. GST-tagged proteins were prepared in E. coli, affinity-purified, and digested where indicated with thrombin. About 200 ng of each protein was then size-fractionated using a 12% SDS-PAGE gel and visualized by staining with GelCode Blue dye (Pierce). Up to four amino acid substitutions were introduced into the protein (S131R-THCR) with little apparent effect on yield or purity.

the proteins beforehand with metal salts had no effect on the activity (data not shown). We concluded that at least four, and probably more, mutations have accumulated over the evolution of the SFV S131R gene, and these multiple mutations render the encoded polypeptide inactive as a superoxide dismutase.

SFV SOD Homolog Binds Zinc but Not Copper—Graphite furnace atomic absorption spectroscopy was used to analyze the copper and zinc content of the purified recombinant proteins (Fig. 4). These experiments can be complicated by the presence of trace levels of adventitious metals, so care was taken to exhaustively dialyze the recombinant proteins against metal-free buffers and thus eliminate any weakly associated copper or zinc. In addition, controls were performed that readily detected ~1 mol each of Cu and Zn per mole of dialyzed bovine Cu,Zn-SOD, and we found there was no significant contribution to the metal content from the GST domain (cleaved or not). It should be noted that we also explored several alternative methods of sample preparation, including varying the copper and zinc content of the bacterial media. The metal content of the SFV proteins seemed quite insensitive to such experimental perturbations and this gives us some confidence that the results reported are not especially sensitive to vagaries in the method of protein preparation. These studies showed that wild-type SFV SOD bound ~1.3 mol of zinc per mole of SOD polypeptide, and this quantity of metal was largely unaffected by introducing S131R-T, S131R-TH, and S131R-THC substitutions into the polypeptide. In contrast to the bovine Cu,Zn-SOD control, the Leporipoxivirus proteins bound significantly less copper than zinc, with most of the protein forms binding ~0.2 mol of copper per mole of protein. Although the poor affinity for copper could readily explain why most of these protein species lacked dismutase activity (Fig. 3), it is notable that the S131R-THCR protein also lacked activity even though a significant portion of the protein was seemingly isolated in a Cu-Zn metallated form. The S131R-THCR protein bound both metals, although, unlike bovine Cu,Zn-SOD, it bound less than stoichiometric quantities of each. We detected ~0.35 mol of zinc and ~0.4 mol of copper per mole of protein (Fig. 4). An attractive explanation for these properties of the quadruple mutant protein is that copper may still not be incorporated properly into the active site. Instead it could be displacing zinc from the zinc-binding site, and this anomalous binding could explain the lack of activity.

Leporipoxviruses Cu,Zn-SOD Homologs Form Stable Complexes with Cellular CCS. The experiments described above showed that Leporipoxivirus SOD homologs have retained the zinc-binding property of cellular Cu,Zn-SODs. MYX and SFV SOD-like proteins can also form homodimers (16) much like their cellular counterparts. These observations suggested that the poxvirus proteins might have partially conserved the metal and protein-binding properties of Cu,Zn-SODs even though they may not have retained the catalytic activity over the course of viral evolution. We were thus curious to determine whether SFV and MYX proteins shared a third property of cellular Cu,Zn-SODs; the capacity to form stable complexes with copper chaperones for SOD (CCS). A combination of “GST pull-down” and co-immunoprecipitation studies showed that CCS is another binding partner both in vivo and in vitro.

In the first set of experiments we co-infected BGMK cells with two recombinant vaccinia viruses and prepared a cell-free extract. Vaccinia strain VMM5.2 encoded the SFV S131R gene under the regulation of a T7 promoter and, in the presence of a helper virus encoding T7 RNA polymerase (VTF7.5) caused the expression of large quantities of native SFV SOD (16). Extracts were prepared from these cells and incubated with a fusion protein consisting of GST-linked to CCS and immobilized on glutathione-Sepharose beads. The bound protein was eluted with reduced glutathione and separated using SDS-PAGE, and a Western blot was performed using a monoclonal antibody specific for SFV SOD (Fig. 5). The GST-tagged CCS readily extracted an 18-kDa SFV SOD from vaccinia-infected cell lysates. No trace of the 18-kDa polypeptide was recovered from mock infected cell extracts, nor did GST alone bind to SFV.
SOD. SFV SOD thus appeared to form complexes with CCS.

To further investigate the specificity of this interaction, polyclonal antisera directed against CCS domains I and III, or monoclonal antibodies directed against SFV SOD, were immobilized on protein A-agarose beads and incubated with vaccinia-infected BGMK cell lysates containing recombinant SFV SOD. Any bound proteins were eluted from the beads and separated by SDS-PAGE, and a Western blot was performed using different antibodies. Although BGMK cells have a monkey origin, the \( \alpha \)H9251 human CCS antibodies immunoprecipitated an appropriately sized monkey CCS peptide from both mock and virus-infected cell extracts (Fig. 6, middle panel). The \( \alpha \)CCS antibodies co-immunoprecipitated SFV SOD from a virus-infected cell extract but not from mock-infected cell extracts (Fig. 6, top). In the reciprocal experiment, the \( \alpha \)SFV SOD monoclonal antibody immunoprecipitated SFV SOD from virus-infected cell extracts (Fig. 6, top) and co-immunoprecipitated the 30-kDa monkey CCS from the same sample (Fig. 6A, middle). BGMK cell CCS was not detected in precipitates obtained from mock infected cell extracts, thus demonstrating the specificity of the \( \alpha \)SFV SOD monoclonal antibody. Oddly, more CCS was detected in virus-infected cells than in mock infected

**Fig. 3.** Superoxide dismutase activity of wild-type and mutant forms of S131R protein. SOD activity was determined by measuring the enzymatic interference in the reduction of cytochrome c in a reaction containing hypoxanthine and xanthine oxidase. Panel A shows a comparison of the activities of wild-type SFV SOD protein and bovine Cu,Zn-SOD. The S131R gene product displays only \( \sim 1\% \) of the SOD activity of a bona fide cellular Cu,Zn-SOD. Panel B shows that introducing up to four amino acid substitutions into S131R still does not restore SOD activity. Both intact (open bars) and thrombin-cleaved (gray bars) GST fusion proteins were assayed to show that removing the GST domain did not alter the activity.

**Fig. 4.** Metal content of wild-type and mutant forms of SFV SOD. Affinity-purified GST fusion proteins were dialyzed extensively at 4 °C against a Chelex-treated 2.5 mM phosphate buffer prior to metal analysis. Except for the SOD-THCR mutant, the zinc contents of the other forms of SFV SOD are comparable to that of bovine Cu,Zn-SOD. None of the SFV SOD species bound copper with a stoichiometry comparable to bovine Cu,Zn-SOD.
cells (compare lanes 1 and 2). We do not know the explanation for this observation, but it seemed to be a reproducible feature of vaccinia virus infection.

Several controls served to validate these methods. The recombinant SFV SOD is highly expressed using T7-based vaccinia vectors, so to exclude the possibility that we were observing a nonspecific binding reaction we substituted an M131R knockout virus (16) was available for use as a control. The aSFV SOD monoclonal antibody for the aCCS antibody and observed no recovery of SFV SOD or CCS (data not shown). We also looked for an interaction in cells infected with myxoma virus where the level of expression of MYX SOD is expected to more closely resemble a normal infection. We chose to use myxoma virus for these studies, because MYX SOD is nearly identical to SFV SOD, the two proteins share 94% amino acid identity, and a myxoma ΔM131R knockout virus (16) was available for use as a control. The aSFV SOD monoclonal antibody cross-reacted with MYX SOD in myxoma-infected cells (Fig. 7, top) and the antibody co-immunoprecipitated monkey cell CCS from the sample (Fig. 7, bottom). The same aSFV SOD antibody did not precipitate CCS from cells infected with MYX ΔM131R deletion virus. In the reciprocal experiment, the aCCS antibodies immunoprecipitated CCS from all of the samples (Fig. 7, bottom).

The distribution of the myxoma protein was nearly identical to SFV SOD (the two proteins share 94% amino acid identity), and a myxoma ΔM131R knockout virus (16). We concluded that, if Leporipoxvirus Cu,Zn-SOD homologs might be exerting their effects on the copper chaperone. The distribution of the myxoma protein was investigated using confocal immunofluorescence microscopy and the aSFV/MYX SOD monoclonal antibody. Preliminary experiments (not shown) detected a speckled staining pattern suggestive of mitochondrial involvement. This was confirmed using a double-labeling strategy where infected cells were stained with both the aSFV/MYX SOD monoclonal antibody.
and a mitochondrial-specific dye (Fig. 9). Although some MYX SOD antigen was distributed throughout the cell by 24 h post-infection, a substantial portion of the Alexafluor (green) aMYX SOD label was seen to overlap with the MitoTracker (red) mitochondrial label (Fig. 9), and this co-localization was observed throughout the cross-sections imaged (data not shown). No SOD antigen was detected in cells infected with the ΔM131R knockout virus or in mock infected cells. We also attempted to localize cellular CCS using the αCCS polyclonal antibody. Unfortunately this antigen-antibody combination produced a weak signal and high background in BGMK cells, and the high gain needed to detect the signal, rendered suspect the interpretation of double-labeling experiments (data not shown). Nevertheless it was clear that the interactions characterized above, between viral Cu,Zn-SOD homologs and cellular CCS, could potentially occur in organelles like the mitochondria.

**Purified Mitochondria Also Contain MYX SOD**—To further investigate the distribution of viral SOD in infected cells, we used cell fractionation methods and Nycodenz density gradient centrifugation to isolate different organelle fractions, including highly purified mitochondria (23). Mitochondria obtained from the 25/30% Nycodenz interface were shown, using Western blots and appropriate protein markers, to be substantially free of contamination by cytosolic, microsomal, and peroxisomal debris (Fig. 10). The α-S131R monoclonal antibody still detected similar amounts of the MYX-SOD antigen in all of the fractions isolated from wild-type virus-infected cells. While these methods it is difficult to be certain that proteins aren’t lost through diffusion from organelles during their fractionation. However, considering the relative amounts of each fraction recovered from the cells and loaded on these gels, we estimated that about 2% of the viral SOD was present in the mitochondrial fractions. No MYX SOD was detected in cells infected with the ΔM131R virus, whereas the presence or absence of MYX SOD had no obvious effect on the amount of cellular Cu,Zn-SOD detected in the mitochondria isolated from cells infected with wild-type or ΔM131R knockout viruses.

**DISCUSSION**

Poxviruses encode a variety of proteins that serve to inhibit the activities of host antiviral defenses. Interestingly, many of these virus genes encode homologs of cellular proteins, and understanding what feature(s) of these cellular orthologs have been retained by the viral protein often provides insights into its function. A good example is the many truncated TNF-receptor mimics encoded by poxviruses, which typically retain the TNF-binding properties of their cellular homologs but lack the signaling and transmembrane domains (25). These proteins...
function as decoys, interfering in the TNF-mediated signaling that can kill infected cells. Poxviruses also encode a number of chemokine binding proteins, which, by binding a wide assortment of chemokines, interfere in the migration of leukocytes to sites of infection or inflammation (26). Leukocytes and macrophages use reactive oxygen species to prime apoptosis or to kill infected cells outright, and it is thus not too surprising that poxviruses might encode homologs of the Cu,Zn-SODs that play a key role in reactive oxygen species metabolism.

Poxviruses are rather genetically stable pathogens in part because they encode proofreading DNA polymerases (27). Yet this work and other data (13, 16) show that, although divergent evolutionary processes may have retained two remarkably conserved forms of SOD homologs among several genera of poxvirus (Fig. 1), these processes have not favored retention of the dismutase function. Indeed, at least four (and probably more) amino acid substitutions have long rendered SFV and MYX Cu,Zn-SOD homologs catalytically inert. These mutations include substitutions that interfere in copper binding (Fig. 4), and this would be expected to have a significant effect on activity given the role of Cu(II–Cu(III) redox reactions in catalysis. That these are not the only catalytically inactivating mutations is suggested by the fact that, although the copper-binding deficiency can be partially overcome in multiply modified forms of the protein (Fig. 4), this alteration does not restore any catalytic activity (Fig. 3). SFV SOD was observed to retain the Zn-binding properties of its cellular homologs (Fig. 4), whereas all of the Orthopoxvirus Cu,Zn-SOD homologs have almost certainly deleted the Zn-binding domain (Fig. 1). We suggest that the zinc atom most likely serves the same non-essential structural role in stabilizing SFV SOD as it does stabilizing cellular Cu,Zn-SOD and that this feature was lost at some point in the evolution of the Orthopoxvirus proteins.

For what purpose might a poxvirus encode such peculiar modified forms of Cu,Zn-SOD? SFV and MYX SOD homologs don’t form heterodimeric complexes with cellular Cu,Zn-SODs (Fig. 6) or affect the level of protein (Fig. 8), but they do share with their cellular Cu,Zn-SOD homologs the capacity to bind cellular copper chaperone for SOD (Figs. 5–7). We suggest that poxvirus Cu,Zn homologs are protein decoys that have retained a selective capacity to bind to cellular CCS, and in this way they can interfere with CCS-catalyzed metallation reactions. This competition between virus and cellular SODs for CCS would decrease the intracellular pool of fully metallated Cu,Zn-SOD and hence cause a decline in dismutase activity even though levels of Cu,Zn-SOD antigen remain static (Fig. 8). Viral SOD homologs are produced and packaged in the virion in abundance (13, 16), and so if Cu,Zn-SOD activity is slowly turning over in virus-infected cells, such a model could explain why one sees a gradual, M/S131R-dependent, reduction in the activity of Cu,Zn-SOD over the course of viral infection (16). The peculiar structures of poxvirus SOD homologs are also compatible with this model. When one looks at the structure of SFV and MYX SODs (Fig. 1), it is noteworthy that many amino acids are conserved, which in the homologous yeast protein mediate an interaction with yeast CCS. Moreover, cysteine C67 is remarkably well positioned to participate in the formation of an intermolecular disulfide bond bridging a viral SOD homolog to the CCS (11). Although this disulfide bond is probably not essential for stable complex formation (11), the presence of this residue may be one more factor favoring formation of dead-end complexes at the expense of properly metallated cellular Cu,Zn-SOD.

The fact that Leporipoxvirus SOD homologs cannot bind copper would not preclude formation of these heterodimeric complexes, because several studies have observed interactions between copper-deficient mutant SOD proteins and CCS (10, 20). Indeed, the inability to incorporate copper in the viral partner may be another important stabilizing factor that effectively traps the two proteins in an inactive state, and it is noteworthy that the complexes involving viral SOD homologs are seemingly more stable than are those formed between CCS and cellular Cu,Zn-SOD. (We observed that CCS can be used to co-immunoprecipitate SFV SOD but not cellular Cu,Zn-SOD (Figs. 5 and 6).) Such a regulatory model is not entirely new. Several years ago, Schmidt et al. (28) proposed that cells could regulate the level of superoxide dismutase activity by having that activity depend upon a coordinated interaction between two separate proteins. Poxviruses may simply have interposed a SOD mimic into this normal cellular process, and we are currently generating a system that can directly measure the effect of S131R on the chaperone activity of CCS.

The inhibitory properties of these proteins suggest that leporipoxviruses gain some benefits from interfering with the dismutation of superoxide radical and disrupting cellular redox homeostasis. How might increasing the intracellular concentration of \( \text{O}_2^- \) benefit a virus? One can imagine several advantages, however, the most attractive explanation relates to the fibroanthosarcoma-like tumors formed by MYX and SFV in natural infections (29). Superoxide can have subtle effects on cells, depending upon the concentration, but it is noteworthy that decreasing \( \text{O}_2^- \) concentrations can create a “pro-apoptotic” environment, whereas increasing the concentration of superoxide anion can inhibit Fas-mediated apoptosis and stimulate proliferation (30, 31). In this regard it is especially striking that some portion of the MYX SOD protein co-localizes with the mitochondrial compartment. It has been speculated that it is the unmetallated form of cellular Cu,Zn-SOD that is transported into the mitochondrial intermembrane space where it is then trapped by a CCS-catalyzed copper transfer reaction (32). If this is correct, then targeting a competing viral SOD homolog to the same location might be expected to significantly alter the concentration of active Cu,Zn-SOD within the mitochondrial compartment and perhaps have dramatic effects on apoptotic processes originating in the mitochondria. It thus makes perfect biological sense for these viruses to encode a protein capable of increasing \( \text{O}_2^- \) concentrations in virus-infected cells, because that would help promote the extensive cellular proliferation so characteristic of Leporipoxvirus infections. It should be noted that we could not previously detect any obvious effects of deleting \( M131R \) on the virulence of myxoma virus (16), but a gene promoting tumor growth might not have been amenable to study using this animal model. Myxomatosis is a disease peculiar to domestic rabbits (Oryctolagus cuniculus) and causes a rapidly lethal systemic infection and bacteremia associated with profound immunosuppression. However, in its natural host (Sylvilagus sp.) myxoma causes a very different self-limiting disease associated with large tumor-like growths (29), and only under these disease conditions might the subtle effects of a gene affecting tumorigenesis be seen. SFV causes a self-limiting disease in domestic rabbits that much more closely replicates the natural disease course in its host, Sylvilagus floridanus, and we are currently investigating the prediction that SFV \( S131R \) plays a role in virus-induced tumorigenesis using this alternative animal model.

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