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Biochemistry

Glutaredoxin homolog encoded by vaccinia virus is a virion-associated enzyme with thioltransferase and dehydroascorbate reductase activities

(disulfide reduction/glutathione/transhydrogenase)

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ABSTRACT **Glutaredoxins (GRXs), also known as thioltransferases, use glutathione as a cofactor for reduction of disulfides in prokaryotes and eukaryotes. We demonstrate that the vaccinia virus O2L open reading frame encodes a functional GRX, as predicted by Johnson *et al.* [Johnson, G. P., Goebel, S. J., Perkus, M. E., Davis, S. W., Winslow, J. P. & Paoletti, E. (1991) *Virology* 181, 378–381] from sequence homology. The 12-kDa protein product of the O2L open reading frame was synthesized after viral DNA replication, coincident with a major increase in cytoplasmic glutathione-dependent thioltransferase activity. The protein was associated with purified vaccinia virions and was not released by treatment with a nonionic detergent unless dithiothreitol was added. The virion-derived protein, as well as a recombinant form expressed in *Escherichia coli*, exhibited thioltransferase and dehydroascorbate reductase activities indicative of a functional GRX. The postreplicative synthesis of vaccinia virus GRX and its association with virions suggest that the enzyme may have novel roles in the virus growth cycle.**

Glutaredoxins (GRXs), also called transhydrogenases or thioltransferases, are heat-stable proteins of approximately 12 kDa that contain a glutathione (GSH)-dependent, redox-active disulfide bond. GRX genes (*grx*) have been cloned from *Escherichia coli* (1), yeast (2), and mammals (3, 4). The protein was initially identified as an alternative hydrogen donor for the reduction of ribonucleotide reductase in an *E. coli* mutant deficient in thioredoxin (TRX), an unrelated disulfide–dithiol protein (5, 6). Oxidized GRX is reduced by GSH, which is regenerated by NADPH and GSH reductase, whereas oxidized TRX is reduced directly by NADPH and TRX reductase (7, 8).

Not all GRXs have demonstrable ribonucleotide reductase cofactor activities (9), and GRXs have been implicated as regulators of diverse cellular activities (10, 11). In addition, GRXs and TRXs are used by bacteriophages. Bacteriophage T4 encodes a GRX and a ribonucleotide reductase that act cooperatively (12). *E. coli* TRX is an essential subunit of bacteriophage T7 DNA polymerase (13) and also is involved in the assembly of filamentous phage (14).

There has been no evidence that TRXs or GRXs have specific roles in animal virus infections. Recently, however, vaccinia virus was reported to contain an open reading frame (ORF) that potentially encodes a 12-kDa protein with significant similarity to GRXs (15, 16). Vaccinia virus, a well-characterized member of the poxvirus family, replicates in the cytoplasm of infected cells and encodes numerous enzymes involved in DNA replication and transcription (17), including ribonucleotide reductase (18, 19). In the present study, we demonstrate that the vaccinia virus O2L ORF encodes a functional GRX with thioltransferase and dehydro-L-ascorbate (DHA) reductase activities. The protein is synthesized after the onset of DNA replication and is associated with purified virions, suggesting that it may have novel roles in the virus growth cycle.

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MATERIALS AND METHODS

Growth of Virus and Cells. Vaccinia virus was propagated in HeLa cells and titered by plaque assay on BSC-1 cells as described (20).

DNA Cloning and Sequencing. A segment of the genomic DNA of vaccinia virus strain WR, between previously sequenced *Hin*dIII E (21) and I (22) fragments, was amplified by polymerase chain reaction (PCR, Perkin–Elmer/Cetus). The primers BA-E (GCCTTGACTGCAGTCTCATTAATTAACCATTCCAAGTCA, *Pst* I site underlined) and BA-I (TTGTTTGGTCGACTTAAATGGCGGAATTTGAAGATCAACT, *Sal* I site underlined) were designed on the basis of known *Hin*dIII E and I sequences, respectively, that are located immediately outside of the target sequence. The amplified DNA [1.7 kilobase pairs (kbp)] was purified by 0.8% agarose gel electrophoresis, digested with restriction enzymes *Pst* I and *Sal* I (Boehringer Mannheim) and cloned into pBluescript SK (Stratagene) to form pVH(P/O). The nucleotide sequence of pVH(P/O) was determined by double-strand sequencing with Sequenase (United States Biochemical) and synthetic oligonucleotide primers.*

Preparation of Recombinant Protein and Antiserum. For expression of the O2L ORF in *E. coli*, the entire protein coding sequence was amplified by PCR from plasmid pVH(P/O) by using primers GRX-F(AATATTCATATGGCCGAGGAATTTGTACAACAAAGG, *Nde* I site underlined) and GRX-R (AATTCCGGATCCATGAACATGTCGGCGACATGATTA, *Bam*HI site underlined). The amplified DNA was gel-purified, digested with *Nde* I and *Bam*HI, and cloned in expression vector pET3c (23) to construct pET-(GRX). The *E. coli* strain BL21(DE3), a lysogen carrying the gene for the bacteriophage T7 RNA polymerase under the control of the inducible P_L promoter was transformed with pET(GRX) and induced with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 3 hr. The recombinant protein was purified by sodium dodecyl sulfate/16% polyacrylamide gel electrophoresis (SDS/PAGE) in an *N*-[tris-(hydroxymethyl)methyl]glycine (Tricine) buffer system (24) and eluted from the gel as described (25). From 40 ml of induced culture, approximately 200 μg of purified protein was obtained. The protein (100 μg) was emulsified with Freund's

Abbreviations: araC, cytosine arabinonucleoside; DHA, dehydro-L-ascorbate; DTT, dithiothreitol; GRX, glutaredoxin; GSH, glutathionine; IPTG, isopropyl β-D-thiogalactopyranoside; NP-40, Nonidet P-40; ORF, open reading frame; TRX, thioredoxin.

*The sequence whose expression is reported in this paper has been deposited in the GenBank data base (accession no. M76472).

adjuvant and injected into a New Zealand White rabbit that received two boosts (50 μg each) and was bled 5 weeks after the initial injection and thereafter.

Enzyme Assay. GSH reductase-coupled spectrophotometric assays were performed essentially as described (26). Typically, a 500-μl reaction mixture contained 100 mM sodium phosphate at pH 7.5, 1 mM EDTA, 0.3 mM NADPH, 1 mM GSH, 2 units of GSH reductase (Sigma), and appropriate dilutions of the GRX fractions. The reaction was initiated by addition of one of the following substrates: 0.5 mM 2-hydroxyethyl disulfide (Aldrich) or 2.5 mM L-cystine for the thioltransferase assay, or 1 mM freshly made DHA (Fluka) for the DHA reductase assay. A decrease in the absorbance of NADPH was measured at 340 nm starting 30 sec after addition of substrate and for the following 2 min at room temperature. One unit was defined as the amount of enzyme catalyzing oxidation of 1 nmol of NADPH per min.

Western Blotting and Immunoprecipitation. SDS/PAGE was carried out in a Tricine buffer system (24) except when stated otherwise. Western blotting was performed and the immune complex was detected with ^{125}I-labeled staphylococcal protein A (^{125}I-protein A; Amersham) as described (21). Immunoprecipitation was performed as before (25) except that cells were labeled with [^{35}S]cysteine (Amersham) and cell lysis and incubation with antibody were carried out in buffer containing 100 mM NaCl, 100 mM Tris·HCl at pH 7.5, and 0.5% Nonidet P-40 (NP-40).

Purification of Recombinant GRX. The following steps were carried out on ice or at 4°C. An induced bacterial culture (400 ml) was centrifuged at 6000 × *g* for 10 min and the cell pellet was resuspended in 20 ml of buffer A [50 mM Tris·HCl, pH 7.4/10 mM EDTA/2 mM dithiothreitol (DTT)/0.5% Triton X-100, 0.5 mM phenylmethanesulfonyl fluoride/10% (vol/vol) glycerol and lysozyme at 0.5 mg/ml]. Cells were lysed for 30 min with brief sonications to aid protein extraction. Insoluble material was removed by centrifugation at 20,000 × *g* for 30 min, and solid ammonium sulfate was added to 20% saturation with stirring for 20 min. After centrifugation for 30 min at 15,000 × *g*, the supernatant was placed in a fresh tube, ammonium sulfate was added to 80% saturation, and the mixture was stirred for 1 hr. The suspension was centrifuged for 30 min at 15,000 × *g* and the precipitate was resuspended in buffer B (50 mM Tris·HCl, pH 7.8/1 mM EDTA/2 mM DTT/0.01% NP-40/10% glycerol) and applied to a gel filtration column of Sephacryl S-200 (Pharmacia) that had been equilibrated with buffer B. Small samples of the proteins in the effluent fractions were analyzed by SDS/PAGE and stained with Coomassie brilliant blue. The fractions containing the recombinant protein were pooled and dialyzed in buffer B. The sample was applied to an S Sepharose column (Pharmacia), to which most of the recombinant protein did not bind. The flow-through fraction was then applied to a Q Sepharose column (Pharmacia), and bound proteins were eluted with a 0–0.7 M NaCl gradient in buffer B.

RESULTS

DNA Sequence Analysis. This study was initiated to bridge a sequence gap between the *Hin*dIII E and I fragments of the genome of vaccinia virus, strain WR. Direct amplification of viral genomic DNA by PCR was employed as the cloning strategy to ensure the continuity of the unsequenced DNA with its flanking sequences. Thus, a DNA segment encompassing the *Hin*dIII P and O fragments was amplified by using PCR primers derived from the known *Hin*dIII E and I sequences. The amplified DNA was approximately 1.7 kbp in length, consistent with earlier restriction mapping results (22). Four independent clones were sequenced, as a precaution for the possible occurrence of mutations during amplification, but no differences were noted. The entire sequence of the *Hin*dIII P and O fragments comprised 273 and 1442 bp, respectively. Translation of the nucleotide sequence in each of the six possible reading frames located one complete ORF (named O2L) within the *Hin*dIII O fragment. The ORF O2L was flanked by two ORFs (O1L and I1L), of which only part were contained within the *Hin*dIII P and O sequences.

Subsequent to the above sequence determination of the WR strain of vaccinia virus, the genomic sequence of the Copenhagen strain was reported (15). Comparison of the corresponding sequences of the two vaccinia virus strains indicated only 8 nucleotide differences in the *Hin*dIII P and O segments, of which only 2 caused amino acid substitutions (Ala in position 87 of ORF O1L and Val in position 103 of ORF I1L of the Copenhagen strain were Ser and Ala, respectively, in WR). GenBank searches with the putative O2L product indicated approximately 29% and 40% amino acid identities with prokaryotic and eukaryotic GRXs, respectively, as previously reported (16).

Expression of the O2L ORF. The O2L ORF was expressed in *E. coli* and antiserum to the recombinant protein was prepared. The antiserum was used to study the synthesis of the O2L protein in virus-infected HeLa suspension cells. Proteins extracted with 0.5% NP-40 were analyzed by SDS/PAGE and immunoblotting. An immunopositive band was detected at 8 hr after infection and increased in intensity at later times, whereas none was detected in extracts of uninfected cells or in cells infected for 2 or 4 hr (Fig. 1*A*). The migration of the protein during polyacrylamide gel electro-

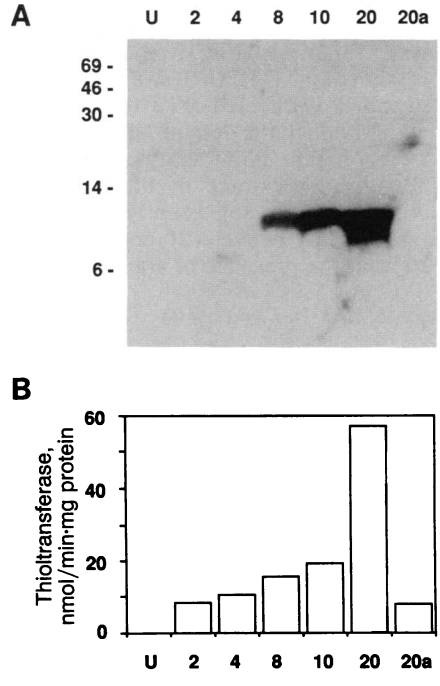

FIG. 1. Expression of the O2L ORF and induction of thioltransferase activity in vaccinia virus-infected cells. (*A*) Virus-infected HeLa suspension cells were harvested at the indicated times and proteins were extracted with 0.5% NP-40 (0°C, 20 min). Proteins (from 5 × 10^6 cells per lane) were separated by SDS/PAGE on a 16% gel in Laemmli buffer (27), electrophoretically transferred to a nitrocellulose membrane, probed with antibody prepared against recombinant O2L protein, and developed with ^{125}I-protein A. An autoradiograph is shown. Lanes: U, uninfected cells; 2–20, hr after infection; 20a, 20 hr after infection in the presence of cytosine arabinonucleoside (araC) at 40 μg/ml. Shown on the left are protein size markers in kDa. (*B*) NP-40 extracts of infected cells were assayed for thioltransferase activity with L-cystine as substrate. The protein concentration of the extracts was determined by the Bradford reagent (Bio-Rad) with bovine serum albumin as a standard.

phoresis was consistent with the molecular mass of 12 kDa predicted from the O2L ORF. In the presence of araC, an inhibitor of viral DNA replication, immunopositive protein was not detected even at 20 hr after virus infection. The absence of expression at early times and inhibition by araC is typical of viral intermediate and late class genes, which are transcribed only after viral DNA replication. Late expression of the O2L protein was consistent with an analysis of the RNA transcribed from this region of the vaccinia virus genome (22).

Metabolic labeling was performed to further examine the time of synthesis of the O2L protein. Infected cells were incubated with [^{35}S]cysteine, and the detergent-extracted proteins were immunoprecipitated with antibody to the recombinant O2L protein. SDS/PAGE and autoradiography indicated that synthesis of the O2L protein occurred at 7 hr and later times after infection (Fig. 2), in agreement with the immunoblotting data. Protein labeled from 7 to 8 hr after infection was stable during a 15-hr chase in unlabeled medium (Fig. 2, lane 7c). The latter result also demonstrated that the antibody was in excess, since the amount of immunoprecipitated radioactive protein was not diminished by the unlabeled protein made during the chase period.

To investigate the possible physical association of early viral proteins such as the ribonucleotide reductase subunits of 87 and 37 kDa with the O2L product, infected cells were radiolabeled from 3 to 8 hr after infection. SDS/PAGE of proteins immunoprecipitated with the O2L protein-specific antiserum revealed only a faint band of 45 kDa in addition to the major 12-kDa band (Fig. 2, lane 3-8). No further experiments along these lines were carried out.

Induction of Thioltransferase Activity. Having demonstrated expression of the O2L ORF, we tested crude extracts of uninfected and virus-infected cell cytoplasm for thioltransferase activity. Although the activity of uninfected cells was below the detection limit of our assay, significant thioltransferase was found in the extracts of infected cells (Fig. 1*B*). Thioltransferase was detectable at early times after infection but increased markedly between 10 and 20 hr. The level of activity at 20 hr in the presence of araC was comparable to the 2-hr level in untreated infected cells. The large amount of thioltransferase at 20 hr was consistent with the late expression of the O2L ORF. We suspect that the activity at early times and in the presence of araC represents low amounts of the O2L product brought into the cell by the incoming virus (see below) but not detected by the immunoblot assays.

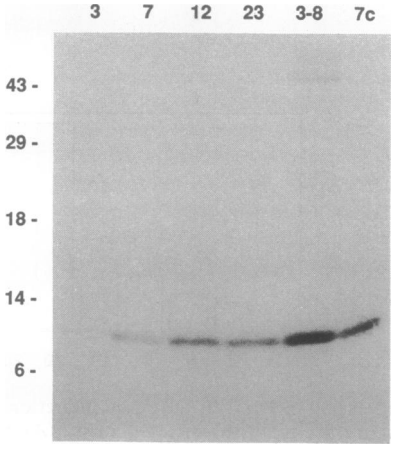

FIG. 2. Time course of synthesis of the O2L protein. Vaccinia virus-infected CV-1 cell monolayers were labeled with [^{35}S]cysteine at 50 μCi/ml (1 Ci = 37 GBq) and lysed with NP-40. The extracts were incubated with antibody made to the O2L protein and beads containing immobilized staphylococcal protein A. The bound proteins were analyzed by SDS/PAGE and autoradiography. Lanes: the first four lanes contain proteins labeled for 1-hr periods starting at 3, 7, 12, or 23 hr after infection; lane 3-8 contains proteins from cells labeled from 3 to 8 hr after infection; and lane 7c contains proteins labeled from 7 to 8 hr after infection and then cultured for 15 more hr in MEM with unlabeled cysteine. Each lane contained proteins from 2 × 10^5 cells. Shown on the left are protein size markers in kDa.

Virion Association of the O2L Protein. The late expression of the O2L protein suggested that it might be incorporated into mature virions. To test this possibility, vaccinia virions were isolated from infected cells by sedimentation through a sucrose cushion, two successive rate zonal sucrose gradient centrifugations, and a CsCl equilibrium density gradient centrifugation. An analysis of the final CsCl gradient is shown in Fig. 3. Association of the O2L protein with the fraction containing virion particles was demonstrated by Western blotting.

Less than 5% of the O2L protein was released into solution by treatment of the purified virions with NP-40 detergent (Fig. 4), which removes the outer lipoprotein coat (28). More than 80% of the O2L protein was extracted, however, by combined NP-40 and DTT treatment of purified virions (Fig. 4), suggesting a subsurface localization of the protein. The extracted O2L protein sedimented more slowly than a 29-kDa marker protein on a glycerol gradient centrifugation, consistent with its release in a nonparticulate form (data not shown).

Partial Purification of the Virion-Associated GRX. The virion-derived O2L protein was partially purified by chromatography on a Q Sepharose column. The peak of the 12-kDa immunoreactive protein eluted at about 200 mM NaCl together with thioltransferase activity (Fig. 5). Silver staining of a gel indicated that the 12-kDa band comprised approxi-

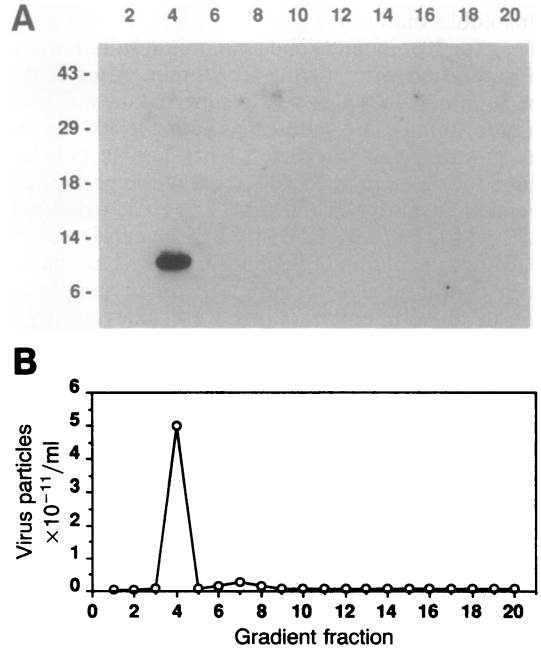

FIG. 3. Association of O2L protein with purified vaccinia virions. Sucrose gradient-purified virions (5 × 10^{11} particles) were centrifuged in a preformed 11-ml CsCl gradient (1.30–1.20 g/ml in 10 mM Tris·HCl buffer, pH 9.0) for 2 hr at 25°C in a Beckman SW41 rotor at 32,000 rpm (180,000 × *g*). Fractions (0.55 ml) were collected from the bottom of the tube. (*A*) Virion proteins in each fraction were solubilized with 2% SDS and 0.75 M 2-mercaptoethanol, separated by SDS/PAGE on a 16% polyacrylamide gel, and immunoblotted as in Fig. 1. An autoradiograph is shown. On the left are protein markers with sizes indicated in kDa. (*B*) Numbers of particles in the fractions, calculated by multiplying the absorbance at 260 nm by 1.2 × 10^{10}.

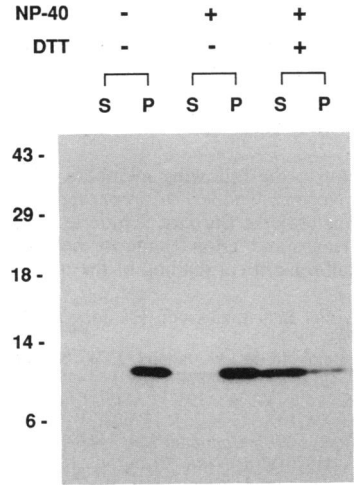

FIG. 4. Release of the O2L protein from virions. Purified virions (1×10^{10} particles) were incubated at 37°C for 1 hr in 50 mM Tris·HCl buffer, pH 8.6 (lanes 1 and 2 from the left), or in the buffer containing 0.5% NP-40 (lanes 3 and 4), or in the buffer containing 0.5% NP-40 and 50 mM DTT (lanes 5 and 6). Soluble fractions were separated from the pellet by centrifugation for 30 min at 4°C in a microcentrifuge at maximum speed. Soluble fractions (lanes 1, 3, and 5) and insoluble pellet (lanes 2, 4, and 6) were boiled in SDS gel loading buffer and electrophoresed in a 16% gel. An autoradiograph of the immunoblot is shown. On the left are protein markers with their size indicated in kDa.

mately 10% of the total protein present in the peak enzyme fractions (data not shown).

Recently, Wells *et al.* (29) demonstrated that pig GRX had DHA reductase as well as thioltransferase activities. Similarly, the partially purified vaccinia virus enzyme also had DHA activity (Fig. 5*B*).

Recombinant GRX. The above results strongly indicated that the vaccinia virus gene homolog encodes a functional GRX. To prove this, the O2L ORF was overexpressed in *E. coli*. The protein coding sequence was inserted into a bacterial expression vector under the control of the bacteriophage T7 gene *ϕ10* promoter. The resultant plasmid pET(GRX) was then introduced into cells harboring the T7 RNA polymerase gene. Induction with IPTG resulted in the appearance of a prominent new polypeptide that migrated on SDS/PAGE at the position expected for a protein of 12 kDa, whereas no such protein was induced in cells transformed with the control plasmid pET3c (Fig. 6). In the absence of IPTG, approximately 0.5 units of thioltransferase per μg of protein was detected, with 2-hydroxyethyl disulfide as the substrate, in a lysate of bacteria transformed with pET(GRX). The thioltransferase activity increased a further 5-fold when the cells were induced with IPTG. Under the same assay conditions, no thioltransferase was detected in extracts prepared from cells that had been transformed with the vector alone.

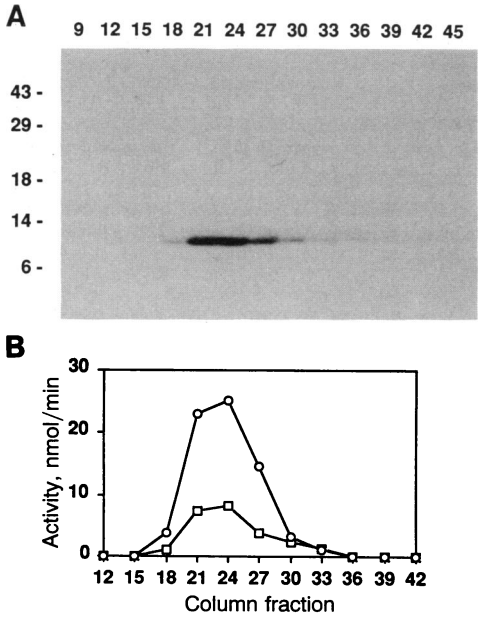

FIG. 5. Purification of the virion-derived GRX. (*A*) Soluble proteins were extracted from 1×10^{13} virion particles by treatment with 0.5% NP-40 and 50 mM DTT, and GRX was purified by Q Sepharose column chromatography. Fractions were analyzed by SDS/PAGE on a 16% gel followed by immunoblotting. An autoradiograph is shown with protein markers on the left. (*B*) Enzyme activity. Fractions were assayed for thioltransferase with 2-hydroxyethyl disulfide as substrate (□) and DHA reductase activities (○).

The soluble recombinant 12-kDa protein was purified by ammonium sulfate precipitation, gel filtration, and cation- and anion-exchange column chromatography steps until only a single band was detected on stained gels (Fig. 7*A*). Thioltransferase and DHA reductase assays were performed with individual column fractions. The enzyme activity profiles correlated well with the amounts of the 12-kDa polypeptide present in the same samples (Fig. 7*B*). The recombinant GRX eluted at approximately 200 mM NaCl, as did the natural viral protein (Fig. 5). The specific thioltransferase activity of the purified vaccinia virus recombinant GRX was 30 units/μg of protein, which was comparable to that of the pig recombinant GRX (30).

DISCUSSION

This study demonstrates the existence of a functional GRX encoded by a virus that infects eukaryotic cells. The vaccinia virus-derived 12-kDa protein, as well as the recombinant protein expressed in *E. coli* and purified to homogeneity, were shown to have thioltransferase and DHA reductase activities. On the basis of these results, and sequence similarity with other members of the GRX family, we suggest that the viral gene be designated *grx*.

Genetic and biochemical studies suggest that some GRXs are cofactors for ribonucleotide reductase (5, 31–33). Indeed, the encoding of both subunits of ribonucleotide reductase by vaccinia virus provides a rationale for the presence of a viral *grx*. If the function of the vaccinia virus GRX is to provide nucleotide precursors for DNA synthesis, the GRX ought to be expressed prior to DNA replication as are thymidine kinase, thymidylate synthetase, ribonucleotide reductase, and DNA polymerase (34). We find, however, that *grx* is

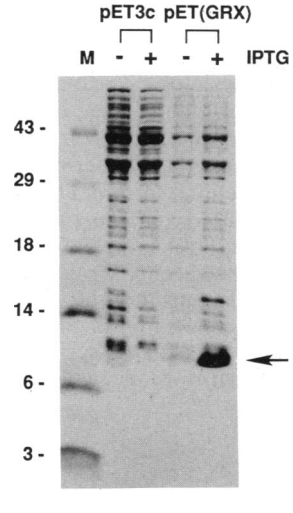

FIG. 6. Expression of the vaccinia virus O2L ORF in *E. coli*. Total bacterial lysate proteins were resolved by SDS/PAGE. A Coomassie brilliant blue-stained 16% polyacrylamide gel is shown. Lanes: M, protein markers with their molecular masses in kDa on the left; pET3c, cells transformed with the pET3c vector and uninduced (−) or induced (+) with IPTG; pET(GRX), cells transformed with pET(GRX) and uninduced (−) or induced (+). Arrow on the right indicates the recombinant protein.

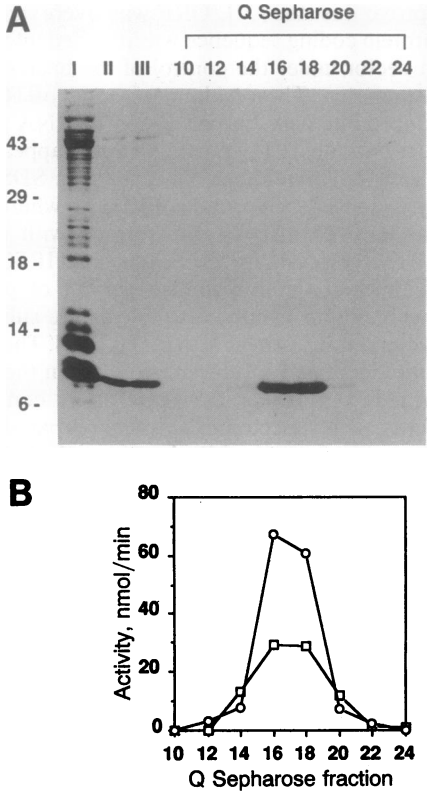

FIG. 7. Purification of recombinant GRX. (*A*) Samples were analyzed by SDS/PAGE. A Coomassie brilliant blue-stained 16% gel is shown. Lanes: I, ammonium sulfate-precipitated material; II, pooled fractions from Sephacryl S-200 column; III, flow-through material from the S Sepharose column; 10–24, fractions from Q Sepharose column. Size of the protein markers in kDa is shown on the left. (*B*) Enzyme activity of the Q Sepharose fractions. □, Thioltransferase activity assayed with 2-hydroxyethyl disulfide; ○, DHA reductase activity.

expressed only postreplicatively. In addition, whereas GRX is associated with infectious vaccinia virus virions purified by successive rate zonal and equilibrium centrifugations, none of the other viral enzymes involved in DNA synthesis are so localized. On the basis of the specific activity of the purified recombinant protein and the amount of thioltransferase extracted from virions with NP-40 and DTT, we estimate that there are 350 molecules of GRX per virion. This number is slightly higher than the copies of other enzymes in vaccinia virus cores (35–38). Although it remains possible that sufficient GRX is brought into the cell by the incoming virus to serve as a ribonucleotide reductase cofactor prior to DNA replication, other roles for the enzyme are likely.

GSH is usually present in millimolar concentrations in the cell cytoplasm, and as the principal physiological reductant, it is likely responsible for the thiol/disulfide balance in the cell (39, 40). Roles for GSH in prokaryotic and eukaryotic cells include the regulation of enzymes via thiol–disulfide conversion, protection of cellular functions from oxidative stress, and storage and transport of sulfur-containing molecules. These activities are mediated by several GSH-dependent enzymes, including GRX. The vaccinia virus GRX might participate in virion assembly, stabilization of virion infectivity in oxidizing environments, uncoating of virions after cell penetration, and activation of the viral transcription system to initiate gene expression. *In vitro*, reducing agents are required to disassemble virions (28), activate transcription (41, 42), and release RNA polymerase from cores (43). The availability of purified, active, recombinant GRX and the potential to mutagenize the vaccinia virus *grx* for *in vivo* studies should allow testing of these and other hypotheses.

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