Complete pathway for protein disulfide bond formation encoded by poxviruses

Tatiana G. Senkevich*, Christine L. White*, Eugene V. Koonin†, and Bernard Moss*‡

*Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, and †National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD 20892

Contributed by Bernard Moss, March 20, 2002

We show that three cytoplasmic thiol oxidoreductases encoded by vaccinia virus comprise a complete pathway for formation of disulfide bonds in intracellular virion membrane proteins. The pathway was defined by analyzing conditional lethal mutants and effects of cysteine to serine substitutions and by trapping disulfide-bonded heterodimer intermediates for each consecutive step. The upstream component, E10R, belongs to the ERV1/ALR family of FAD-containing sulfhydryl oxidases that use oxygen as the electron acceptor. The second component, A2.5L, is a small α-helical protein with a CxxxC motif that forms a stable disulfide-linked heterodimer with E10R and a transient disulfide-linked complex with the third component, G4L. The latter is a thioredoxin-like protein that directly oxidizes thiols of L1R, a structural component of the virion membrane with three stable disulfide bonds, and of the related protein F9L. These five proteins are conserved in all poxviruses, suggesting that the pathway is an ancestral mechanism for direct thiol-disulfide interchanges between proteins even in an unfavorable reducing environment.

Infectious intracellular mature vaccinia virions, consisting of lipoprotein membranes surrounding a DNA-containing protein core, are assembled in the cytoplasm and subsequently wrapped with an additional membrane derived from trans-Golgi or endosomal cisternae before exocytosis (1). Remarkably, some intracellular mature vaccinia virion components have stable disulfide bonds in their cytoplasmic domains (2) that could not be acquired through known cellular pathways, which occur in the relatively oxidizing endoplasmic reticulum (ER) (3, 4). To account for the formation of stable disulfide bonds in the cytoplasmic compartment, we suggested the participation of two vaccinia virus redox proteins, E10R (5) and G4L (6).

E10R is essential for vaccinia virus assembly (7) and orthologs of this protein are present in all sequenced poxviruses (5). Repression of E10R prevented the formation of disulfide bonds in the cytoplasmic domain of L1R, a membrane component of the intracellular form of vaccinia virus, and in the closely related F9L (5). E10R belongs to the ERV1/ALR family of eukaryotic proteins, which contain a conserved CxxC motif implicated in redox function (8). FAD-dependent sulfhydryl oxidase activity has been demonstrated for five members of this family (9–14) and is likely to be present in others. The ERV1/ALR family member ERV2p, an integral membrane flavoprotein of the yeast ER, directly participates in an alternative pathway of disulfide bond formation by using molecular oxygen as the electron acceptor (13). The crystal structure of ERV2p complexed with FAD revealed a novel α-helical fold with a mode of FAD binding distinct from that seen in other FAD-dependent oxidoreductases (15).

G4L, a vaccinia virus thioredoxin homolog, conserved in all poxviruses, has thiol transferase activity *in vitro* (16) and, like E10R, is required for formation of disulfide bonds in L1R and F9L and for assembly of vaccinia virions (6, 17). Repression of E10R prevents thiol oxidation of G4L, whereas the reverse is not true (5, 6). Consequently, G4L is thought to act downstream of E10R in the putative virus-specific redox pathway.

Here we show that A2.5L, a previously uncharacterized vaccinia virus redox protein, represents the missing link between E10R and G4L, and we define the complete virus-specific pathway of disulfide bond formation by detecting disulfide-linked protein heterodimer intermediates for each consecutive step up to the ultimate disulfide-bonded virion proteins. These results indicate that direct transfer of electrons by thiol-disulfide exchange reactions may provide a mechanism of disulfide bond formation even in a highly reducing environment.

Materials and Methods

Expression Plasmids. Vaccinia virus ORFs were amplified by PCR using DNA from the Western Reserve strain as a template and cloned in pGEM-T easy vector (Promega). The adjacent natural promoter or another vaccinia virus strong late promoter sequence was included at the 5′ end of the forward PCR primer. The sequences coding for the hemagglutinin (HA) or V5 tags were included at the 3′ end of the reverse PCR primers. The *L1R* and *F9L* genes were expressed under strong late promoters, namely the P11 promoter (GAATTTCATTTTGTTTTTTTC-TATGCTATAAATG) and a modified synthetic promoter (GTTTTTTTTTTTTTTTTTTTTACTGGATAAATG), respectively; ATG start codons are underlined. E10R, A2.5L, and G4L were expressed under control of their natural promoters or the late P11 promoter as indicated in the figure legends.

Viruses. vE10Ri, vG4Li, and vA2.5Li are inducer-dependent derivatives of the Western Reserve strain of vaccinia virus containing the corresponding genes under the control of a bacteriophage T7 promoter and *lac*-operator regulated by isopropyl-β-D-thiogalactopyranoside (IPTG). Without IPTG addition, the synthesis of the respective proteins was severely depressed. The construction and characterization of vE10Ri and vG4Li have been described (6, 7); the vA2.5Li mutant will be described in detail elsewhere. The vE10R-HA/A2.5L-V5 double recombinant virus was made from the previously described vE10R-HA virus containing the *E10R* gene with a C-terminal HA tag replacing the original *E10R* gene (7). The V5 tag was added to the C terminus of the *A2.5L* gene by using the same procedure as used for construction of vE10R-HA, except that the enhanced green fluorescent protein gene was used as the screening marker.

Transfection of Vaccinia Virus-Infected Cells with Expression Plasmids. BS-C-1 cells in a 24-well plate were infected with five plaque-forming units of virus per cell and 2 h later were transfected with 0.25 μg of plasmid that had been preincubated with 2 μl of Lipofectamine 2000 (Invitrogen) according to the manufactur-

Abbreviations: AMS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid; IPTG, isopropyl-β-D-thiogalactopyranoside; NEM, *N*-ethylmaleimide; TCA, trichloroacetic acid; HA, hemagglutinin; ER, endoplasmic reticulum.

‡To whom reprint requests should be addressed. E-mail: bmoss@nih.gov.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

er's protocol. Where indicated, 100 μg of IPTG per ml was added at 2 h after transfection. The cells were collected by centrifugation at 18 h after infection with or without pretreatment of the cell monolayer with 10% trichloroacetic acid (TCA). Cell pellets were solubilized in nonreducing SDS/PAGE loading buffer (Invitrogen) containing 20 μM *N*-ethylmaleimide (NEM; Sigma) or (4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid) (AMS; Molecular Probes). The pH values of the samples treated with TCA were adjusted by addition of 2 M Tris·HCl, pH 8.5. All lysates were sonicated and boiled.

Western Blot Analysis. Proteins were resolved by SDS/PAGE; 16% Tricine or 10–20% Tris-glycine gels (Invitrogen) were used for small or large proteins, respectively. After PAGE, proteins were transferred to a nitrocellulose membrane, incubated with peroxidase-conjugated anti-HA monoclonal rat high affinity Ab (3F10, Roche Applied Science) or mouse monoclonal anti-V5 Ab (Invitrogen), and detected with a chemiluminescence detection kit (Pierce).

Results

Vaccinia Virus A2.5L Is a Component of a Disulfide Bond Formation Pathway. Our previous studies indicated that the proteins E10R and G4L are components of a common redox pathway. Although proteins acting consecutively in thiol-disulfide transfer form transient disulfide-bonded heterodimers, we were unable to detect an E10R$_{ss}$G4L complex, raising the possibility of an additional redox protein between E10R and G4L. The missing link was suspected to be vaccinia virus-encoded A2.5L because an interaction of this protein with E10R was detected in a global yeast, two-hybrid analysis of vaccinia virus proteins (18). Orthologs of the 9-kDa A2.5L protein are present in all poxviruses, contain a conserved CxxxC or CxxC motif (8), show no detectable sequence similarity to any other proteins, and have an all-α-helical fold based on secondary structure predictions (data not shown).

When a plasmid with a HA epitope-tagged A2.5L ORF regulated by its natural promoter was transfected into cells infected with vaccinia virus, an HA-Ab-reactive protein of the expected size (10 kDa) was detected by SDS/PAGE under reducing conditions (data not shown). In all experiments shown, proteins were analyzed under nonreducing conditions that maintained their redox state. Because molecules of A2.5L-HA with free thiols and molecules with intramolecular disulfide bonds would have similar electrophoretic mobilities, we used the thiol-conjugating agent AMS (molecular mass 0.536 kDa) to distinguish them. Two AMS residues would add 1.072 kDa to the mass of reduced A2.5L but would leave disulfide-bonded A2.5L unchanged. Our detection of two bands by Western blotting with the anti-HA Ab indicated the presence of the reduced (upper band) and the intramolecular disulfide-bonded (lower band) forms of A2.5L-HA in transfected cells infected with wild-type vaccinia virus (Fig. 1). To determine whether oxidation of A2.5L depended on either E10R or G4L, we infected cells with conditionally lethal IPTG-inducible mutants vE10Ri (7) or vG4Li (17). In the absence of IPTG, synthesis of E10R or G4L was severely repressed. Both reduced and oxidized A2.5L were detected when the cells were infected with vG4Li in the presence or absence of inducer or with vE10Ri in the presence of inducer, but only reduced A2.5L was found with vE10Ri in the absence of the inducer (Fig. 1). These results indicated that E10R was required for oxidation of A2.5L, whereas G4L was not.

To determine the role of A2.5L, we constructed a conditionally lethal recombinant virus called vA2.5Li with an IPTG-inducible *A2.5L* gene (to be described in more detail elsewhere). When A2.5L was repressed, E10R and G4L as well as the substrate proteins L1R and F9L were completely reduced, whereas oxidized forms were detected when IPTG was added

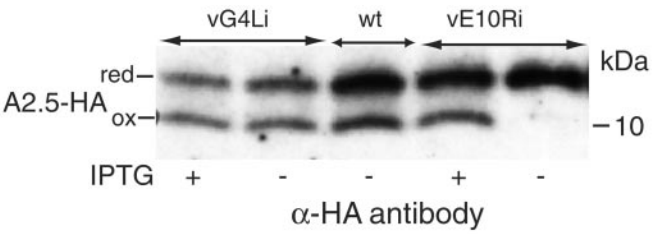

Fig. 1. Effect of E10R and G4L on the thiol-disulfide state of A2.5L. Cells were infected with vG4Li, wild-type (wt) vaccinia virus, or vE10Ri in the presence (+) or absence (−) of IPTG and transfected with a plasmid expressing the *A2.5L* gene with an HA tag regulated by its natural promoter. Samples were lysed in nonreducing SDS/PAGE-loading buffer containing AMS, resolved by SDS/PAGE in a 16% Tricine gel, and analyzed by Western blotting with peroxidase-conjugated anti-HA Ab. The reduced (red) and disulfide-bound, oxidized (ox) forms of A2.5L-HA are indicated.

(not shown) or when an A2.5L expression plasmid was transfected (compare the first and second lanes in each panel of Fig. 2). These results indicated that A2.5L functions upstream of G4L, L1R, and F9L in the disulfide bond formation pathway. However, because E10R and A2.5L were required for oxidation of each other, additional experiments were needed to determine their relative positions in the disulfide bond pathway.

Effects of Cysteine-to-Serine Substitutions in A2.5L. Because E10R belongs to the ERV1/ALR family of FAD-dependent sulfhydryl oxidases that use O_2 as the electron acceptor, we anticipated that it functioned before A2.5L in the redox pathway. If correct, the cysteines of A2.5L should not be needed for oxidation of E10R. In the experiments depicted in Fig. 2, cells were infected with vA2.5Li in the absence of IPTG to repress the synthesis of natural A2.5L and transfected with plasmids that express A2.5L with cysteine to serine substitutions. As predicted, the replacement of one or both cysteines of A2.5L with serine had no effect on E10R oxidation (Fig. 2*A*). In contrast, the substitution of serines for both cysteines of A2.5L resulted in the accumulation of reduced G4L, L1R, and F9L (Fig. 2 *B–D*) consistent with the position of A2.5L upstream of G4L in the redox pathway. Single cysteine to serine replacements in A2.5L severely reduced but did not entirely abrogate oxidation of G4L, L1R, and F9L (Fig. 2 *B–D*). In particular, substitution of the first cysteine of the CxxxC motif allowed a substantial level of L1R oxidation. We will discuss a likely explanation for the activity of single cysteine-substituted A2.5L in a subsequent section. The data presented thus far indicate that the redox pathway is E10R $\rightarrow$ A2.5L $\rightarrow$ G4L $\rightarrow$ L1R (F9L). However, to rule out additional cellular or viral protein components of the redox pathway, it was necessary to demonstrate disulfide-bonded heterodimer intermediates at each step in the transfer of the disulfide bond e.g., E10R$_{ss}$A2.5L, A2.5L$_{ss}$G4L, G4L$_{ss}$L1R, and G4L$_{ss}$F9L.

A2.5L Forms a Stable Disulfide-Linked Heterodimer with E10R. Besides E10R and A2.5L monomers, homodimers of each protein were detected when one was overexpressed relative to the other (data for A2.5L homodimer shown later). Additionally, in all experiments in which both E10R and A2.5L were present, a band with mobility corresponding to the size of their heterodimer ($\approx$23 kDa) was detected (Fig. 2*A*). The putative E10R$_{ss}$A2.5L heterodimer was absent when both cysteines of A2.5L were mutated to serines and greatly diminished when the second cysteine alone was mutated (Fig. 2*A*), suggesting that the link involved the second cysteine of A2.5L. To further characterize the heterodimer, we infected cells with vE10Ri in the absence of inducer to repress natural E10R synthesis and transfected the cells with a plasmid encoding A2.5L containing a C-terminal HA

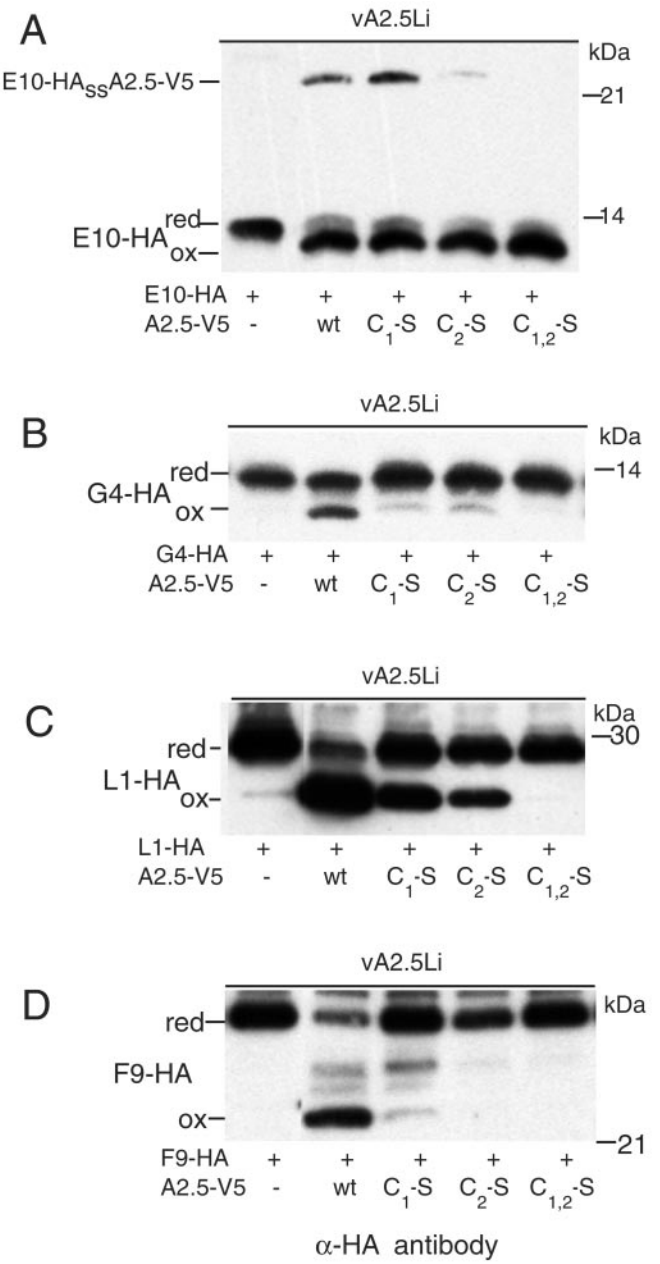

Fig. 2. Effect of A2.5L cysteine-to-serine mutations on the thiol-disulfide state of other proteins. Cells were infected with vA2.5Li in the absence of IPTG and transfected with a plasmid expressing the *E10R* gene with a HA tag regulated by its natural promoter (*A*), the *G4L* gene with an HA tag regulated by its natural promoter (*B*), the *L1R* gene with an HA tag regulated by a strong late P11 promoter (*C*), or *F9L* gene with an HA tag regulated by a modified synthetic strong late promoter (*D*). As indicated in each experiment, we cotransfected a vector plasmid (−) or a plasmid expressing unmutated A2.5L-V5 under control of a strong late P11 promoter (wt), or A2.5L-V5 in which the first (C_1-S), second (C_2-S), or both ($C_{1,2}$-S) cysteines were replaced by serines. Samples were lysed in nonreducing SDS/PAGE-loading buffer containing AMS (*A* and *B*) or NEM (*C* and *D*), resolved by SDS/PAGE in a 16% Tricine gel (*A* and *B*) or a 10–20% Tris-glycine gel, and analyzed by Western blotting with peroxidase-conjugated anti-HA Ab (*A–D*). The reduced (red) and disulfide-bound, oxidized (ox) forms of proteins are indicated.

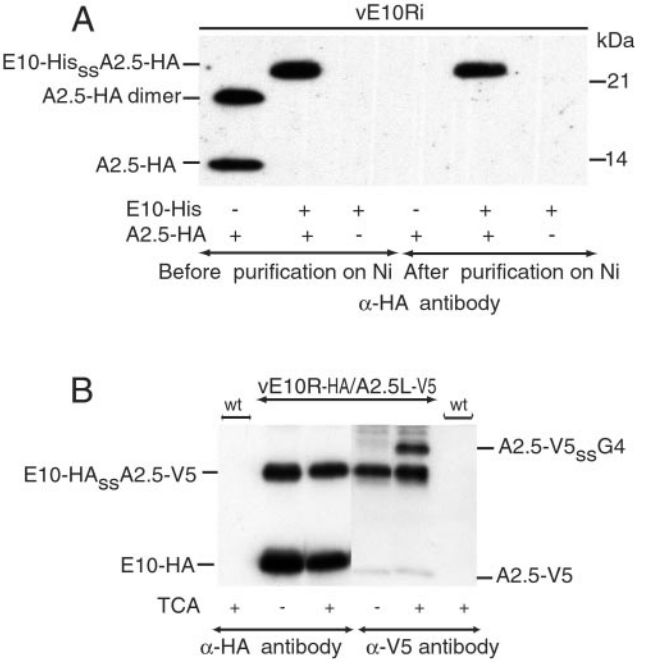

Fig. 3. A2.5L and E10R form a stable disulfide-linked heterodimer. (*A*) Cells were infected with vE10Ri in the absence of IPTG and transfected with one or two plasmids expressing the *E10R* gene with a C-terminal His tag and the *A2.5L* gene with a C-terminal HA tag, both regulated by the strong late P11 promoter. Samples were lysed in nonreducing SDS/PAGE-loading buffer containing NEM, an aliquot of each sample was diluted 1:10 in PBS-1% Triton X-100 and purified on Ni-NTA magnetic beads (Qiagen, Chatsworth, CA). Equal amounts of nonpurified and purified material were resolved in a 10–20% Tris-glycine gel and analyzed by Western blotting with peroxidase-conjugated anti-HA Ab. (*B*) Replicate cell monolayers were infected with a virus containing *E10R* and *A2.5L* genes modified by the addition of a C-terminal HA tag and V5 tag, respectively, or wild-type (wt) virus. Cell monolayers were either treated or untreated with 10% TCA and lysed in the presence of NEM in nonreducing SDS/PAGE-loading buffer. Duplicates of each sample were run in a 10–20% Tris-glycine gel and analyzed by Western blotting with either peroxidase-conjugated anti-HA Ab or anti-V5 Ab.

tag with or without a plasmid encoding E10R containing a C-terminal His tag. When E10R-His was not expressed, bands corresponding to monomer and homodimer forms of A2.5L-HA were detected by nonreducing SDS/PAGE of the total cell extract (Fig. 3*A*). When E10R-His was coexpressed with A2.5L-HA, the putative E10R-His$_{ss}$A2.5L-HA heterodimer replaced the monomer and homodimer species in the whole-cell extracts (Fig. 3*A*). Furthermore, the disulfide-linked complex between E10R-His and A2.5L-HA bound to Ni magnetic beads and was detected with HA Ab, whereas A2.5L-HA by itself did not bind to Ni (Fig. 3*A*).

Because homodimer or heterodimer formation might be driven by overexpression under transfection conditions, we examined the interactions of E10R and A2.5L when synthesized under the control of their natural viral promoters during a virus infection. To this end, we constructed a recombinant vaccinia virus with a HA tag added to the C terminus of the E10R ORF and a V5 tag added to the C terminus of the A2.5L ORF. This recombinant virus replicated normally, indicating that the epitope tags did not compromise the function of either protein. After infection, cell lysates were prepared in the presence of NEM to alkylate any free thiols. The lysates were prepared in duplicate with or without 10% TCA, a pretreatment that keeps thiols protonated and preserves transient disulfide-linked complexes by preventing thiol-disulfide interchange (19), and analyzed by SDS/PAGE. Fig. 3*B* shows different lanes of the same gel developed with either the anti-HA or the anti-V5 Ab. Both Abs detected the same 23-kDa band that corresponded to the E10R-HA$_{ss}$A2.5L-V5 heterodimer. No homodimers of either E10R or A2.5L were present under these conditions. The

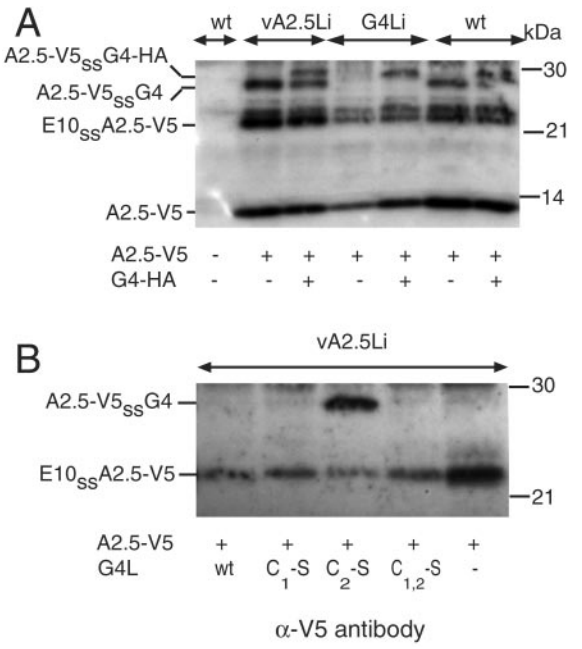

Fig. 4. Detection of a transient A2.5L$_{ss}$G4L heterodimer. (*A*) Cells were infected with vA2.5Li, vG4Li, or wild-type viruses without IPTG, transfected with a plasmid expressing A2.5L-V5, and cotransfected with a vector or a plasmid expressing G4L-HA. Cell monolayers were treated with TCA; proteins were solubilized in nonreducing SDS/PAGE-loading buffer containing NEM, resolved in a 10–20% Tris-glycine gel, and analyzed by Western blotting with peroxidase-conjugated anti-V5 Ab. (*B*) Cells were infected with vA2.5Li without IPTG, transfected with a plasmid expressing A2.5L-V5, and cotransfected with a vector plasmid (−) or a plasmid expressing wild-type G4L or G4L in which the first (C_1-S), second (C_2-S), or both ($C_{1,2}$-S) cysteines were replaced by serines. Samples were lysed in nonreducing SDS/PAGE-loading buffer containing NEM, resolved in a 10–20% Tris-glycine gel, and analyzed by Western blotting with peroxidase-conjugated anti-V5 Ab.

heterodimer was surprisingly abundant, comprising nearly all of the A2.5L-V5 and ≈30% of the E10R-HA. This heterodimer was detected in similar amounts regardless of whether the cells were pretreated with TCA. An additional band of 27 kDa detected with Ab against the V5-tag of A2.5L and seen only in TCA-treated samples (Fig. 3*B*, lane 5), represents the heterodimer between A2.5L and G4L as will be shown below (Fig. 4). Here we want to emphasize that the prevention of the thiol-disulfide exchange by TCA pretreatment was important for detection of the A2.5L$_{ss}$G4L heterodimer but not for the E10R$_{ss}$A2.5L heterodimer.

A Heterodimer of A2.5L and G4L. Additional experiments corroborated the covalent interaction of A2.5L and G4L and demonstrated that expression of E10R was required for this to occur. Cells were infected with vA2.5Li or vG4Li in the absence of inducer to repress synthesis of natural A2.5L or G4L, respectively, or with wild-type vaccinia virus and cotransfected with plasmids expressing either A2.5L-V5 or G4L-HA or both. The lysates were prepared with TCA and analyzed by SDS/PAGE and Western blotting with the anti-V5 (Fig. 4*A*) or anti-HA (data not shown) Ab. Under each of these conditions, the A2.5L-V5 monomer and the E10R$_{ss}$A2.5L-V5 complex were detected (Fig. 4*A*). A higher molecular weight band corresponding to the putative A2.5L-V5$_{ss}$G4L (a complex of A2.5L-V5 with natural untagged G4L) was seen when the cells were infected with the wild-type virus or vA2.5Li but not when G4L was repressed in cells infected with vG4Li. The putative A2.5L-V5$_{ss}$G4L-HA complex, which moved slightly slower than A2.5L-V5$_{ss}$G4L because of the mass of the epitope tag, was seen in cells transfected with the plasmid expressing G4L-HA (Fig. 4*A*). Thus, the complex between A2.5L and G4L was detected only when both proteins were expressed and its mobility was changed in a predicted way when the size of either A2.5L or G4L was increased by the addition of a tag. Significantly, none of these complexes were found when cells were infected with vE10Ri in the absence of inducer (data not shown), indicating that expression of E10R was necessary for oxidation of A2.5L and its subsequent interaction with G4L.

We considered that TCA pretreatment might be required for the detection of the A2.5L$_{ss}$G4L complex to prevent rapid disulfide exchange with the free thiol of G4L. To test this, cells infected with vA2.5Li in the absence of inducer were transfected with plasmids expressing G4L with one or two cysteine to serine substitutions and either unmutated or a cysteine to serine substituted A2.5L-V5. Cells were lysed without acid treatment and the complexes were analyzed by SDS/PAGE. Of the three mutated G4L constructs, only the one retaining the first cysteine of the CxxC motif formed a complex with A2.5L (Fig. 4*B*). This complex was more stable than the one formed with wild-type G4L, as evidenced by the fact that acidification was not required for its detection. An A2.5L-V5$_{ss}$G4L complex also formed with mutated A2.5L, in which the first cysteine was changed to serine, but was not detected when the second cysteine of A2.5L was replaced (data not shown). These results strongly suggest that the disulfide bond in the heterodimer was formed between the second cysteine of the CxxxC motif of A2.5L and the first cysteine of the CxxC motif of G4L.

Detection of Heterodimers Between G4L and L1R or F9L. Intermediates in the transfer of the disulfide bond to the substrate protein were detected by using G4L with a V5 tag on the C terminus and L1R or F9L with a HA tag on the C terminus. Plasmids expressing these proteins were cotransfected into cells infected with vG4Li in the absence of inducer to repress natural G4L, and L1R-HA or F9L-HA were captured with anti-HA Ab conjugated to an agarose matrix. After SDS/PAGE, G4L-V5 covalently associated with L1R-HA or F9L-HA was detected by using the anti-V5 Ab. The G4L-V5$_{ss}$L1R-HA band had the expected molecular weight of 45 kDa (Fig. 5 *Left*); the slightly faster migration of G4L-V5$_{ss}$F9L-HA (Fig. 5 *Right*) was consistent with the smaller size of F9L. The G4L-V5$_{ss}$F9L-HA complex appeared to be comprised of three closely spaced bands, perhaps representing heterodimers associated with different cysteines of F9L-HA. The presence of incompletely resolved higher molecular weight material suggests the existence of additional complexes involving G4L and L1R or F9L proteins. After reduction, only the G4L monomer of 14 kDa was detected with the anti-V5 Ab (data not shown).

If the G4L$_{ss}$L1R and G4L$_{ss}$F9L complexes are formed in the proposed redox pathway, then they should not be detected in the absence of either E10R or A2.5L. Indeed, when plasmids expressing L1R-HA and G4L-V5 were transfected into vA2.5Li-infected cells in the absence of inducer to repress natural A2.5L synthesis, the complexes were detected only when A2.5L-V5 was also cotransfected (Fig. 5 *Center*).

Discussion

We previously provided evidence that the vaccinia virus-encoded proteins E10R and G4L participate in disulfide bond formation (5, 6). Here, we show that A2.5L, a previously uncharacterized vaccinia virus protein with a CxxxC motif and a predicted α-helical structure, is the missing link between E10R and G4L. The complete redox pathway was defined by analyzing the effects of repressing expression of *E10R*, *A2.5L*, and *G4L* genes on disulfide bond formation and of cysteine to serine mutations in corresponding proteins and, most importantly, by

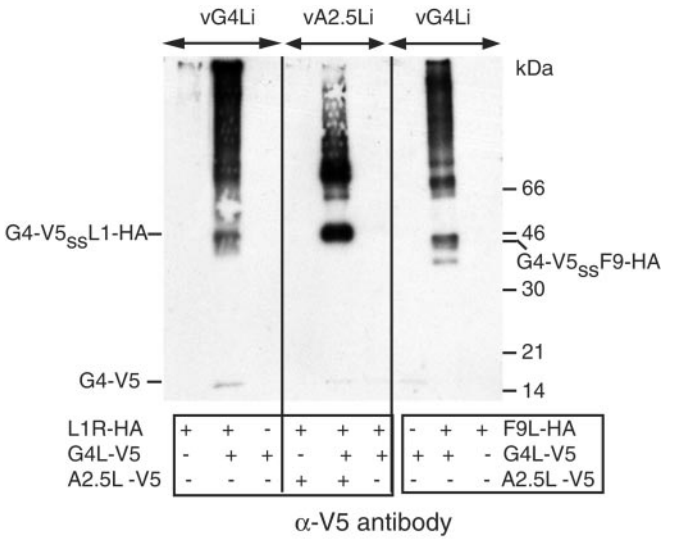

Fig. 5. Detection of transient heterodimers of G4L and L1R or F9L. Cells were infected with vG4Li or vA2.5Li in the absence of inducer, transfected with a vector plasmid or a plasmid expressing G4L-V5 and cotransfected with a vector or a plasmid expressing L1R-HA or F9L-HA. Cell monolayers were treated with TCA; proteins were solubilized in nonreducing SDS/PAGE-loading buffer containing NEM; the pH values of the samples were adjusted to 8.5, and samples were diluted 1:10 in PBS-1% Triton X-100 and immunoprecipitated with anti-HA Ab conjugated to an agarose matrix (Roche Applied Sciences). Proteins reacting with the anti-HA Ab were resolved in a 10–20% Tris-glycine gel and analyzed by Western blotting with peroxidase-conjugated anti-V5 Ab.

capturing specific disulfide-bonded heterodimer intermediates in thiol-disulfide transfer. The pathway consists of three viral redox-active proteins (E10R, A2.5L, and G4L) and two viral substrate proteins (L1R and F9L) with three stable disulfide bonds each (Fig. 6). The detection of disulfide-bonded heterodimers composed of E10R and A2.5L, A2.5L, and G4L, and G4L and L1R or F9L demonstrated the direct interaction between the respective viral proteins in the transfer of disulfide bonds without the involvement of any other protein intermediates.

The viral redox pathway starts with E10R, a member of the ERV1/ALR family of FAD-containing sulfhydryl oxidases. ERV2p, a member of this family involved in an alternative disulfide bond formation pathway in the ER of yeast, was shown to be directly oxidized by molecular oxygen (13). Examination of the recently published crystal structure of ERV2p (15) and the multiple sequence alignment of the ERV1/ALR protein family, showed that amino acids directly involved in FAD binding were highly conserved in E10R as well as throughout the family (data not shown). Consequently, oxygen is likely to be the terminal

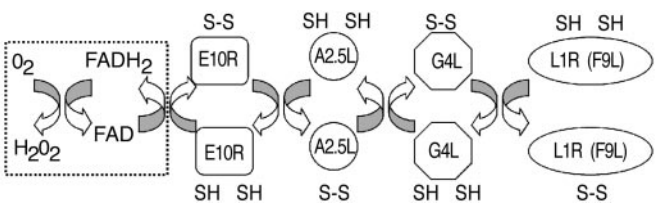

Fig. 6. Vaccinia virus disulfide bond formation pathway. The coupled thiol-disulfide exchange reactions from E10R to the substrate proteins L1R and F9L are depicted. L1R and F9L are each thought to have three intramolecular disulfide bonds. Electron transfers to FAD and eventually to oxygen indicated in the dashed rectangle were not shown experimentally in the vaccinia virus system but were demonstrated for ERV2p. Disulfide bond transfer is thought to occur on the cytoplasmic side of the vaccinia virion membrane.

electron acceptor in the viral redox pathway that directly oxidizes FAD associated with E10R.

The next component, A2.5L, forms disulfide-linked complexes with E10R and G4L, which suggest its intermediate position in the pathway. Surprisingly, A2.5L was required for the oxidation of E10R, although its cysteines were not. This property suggests that the interaction with A2.5L is required for stabilization of an active conformation of E10R or for preventing the formation of E10R homodimers. Several observations suggest that these two proteins do not function as independent oxidoreductases, but rather as an $E10R_{ss}A2.5L$ complex: (*i*) the $E10R_{ss}A2.5L$ complex is more stable than the $A2.5L_{ss}G4L$ complex; (*ii*) most of A2.5L and about 30% of E10R can be recovered as a disulfide-linked complex, an unusually high amount for a transient intermediate, and (*iii*) single-cysteine mutations in A2.5L do not entirely abrogate the oxidation of the downstream components of the pathway, raising the possibility that the heterodimer itself serves as an oxidant under these conditions.

G4L, the next protein in the poxvirus redox pathway, forms disulfide-bonded intermediates with A2.5L as well as with the substrate proteins L1R and F9L. Only a small fraction of G4L was found in each of these complexes. This is compatible with their function as transient intermediates in the disulfide bond formation pathway and differentiates them from the relatively stable $E10R_{ss}A2.5L$ complex. The substrate proteins of the pathway, L1R and F9L, each has three disulfide bonds. This number raises the question of how the correct disulfide pairs are formed and whether an additional isomerase is involved. In *Escherichia coli*, the thiol oxidase DsbA is mostly oxidized, whereas the protein disulfide isomerase DsbC is mostly reduced (20). Because G4Lp is partially oxidized and partially reduced in vaccinia virus-infected cells (6), it may have both oxidase and isomerase functions.

All of the proteins involved in the disulfide bond formation pathway are conserved throughout the poxvirus family, which suggests that the pathway itself is an ancestral mechanism in this group of viruses. The encoding of a complete redox pathway by a virus is unprecedented but may be related to the cytoplasmic site of replication of poxviruses. The redox proteins E10R, A2.5L, and G4L are localized in the cytoplasmic factory regions where virion assembly occurs (7, 17). The cytoplasm is relatively reducing compared to the periplasm and this is unchanged in vaccinia virus infected cells as measured by the ratio of oxidized to reduced glutathione (2). Glutathione, however, is not involved in the poxviral redox pathway because electrons are transferred through covalent protein intermediates. Analogous all-protein pathways function even in the more oxidizing environments, such as the ER of yeast (3, 4) and the periplasm of *E. coli* (21). Although the elaboration of a specific disulfide bond formation pathway by poxviruses was unexpected, it is essential for virus reproduction. Conditional lethal vaccinia virus mutants with inducible copies of any one of the three redox proteins (7, 17) or the substrate protein L1R (22) are blocked at a similar stage in virus assembly under nonpermissive conditions. L1R is a myristoylated protein located on the surface of infectious virions and is an important target of neutralizing Ab (23–25). The location and role of the second substrate protein F9L and the identification of other putative substrates remain to be determined.

A comparison of vaccinia virus-specific redox components to those of other known pathways for disulfide bond formation suggests some interesting analogies. The upstream components of the three known pathways, namely *E. coli* DsbB, and yeast ERO1p and ERV2p, are proteins with two pairs of active cysteines (15, 26–27). In each case, the catalytic pair of cysteines, which interacts with ubiquinone, oxygen, or another non-thiol electron acceptor, forms a CxxC motif. The oxidative equivalents are then transferred to the second pair of cysteines in the same

polypeptide chain, or, in the case of ERV2p, to a second subunit of a homodimer. This second pair of redox-active cysteines does not form any defined motif in DsbB or ERO1p and is present in the unusual CGC configuration in ERV2p. E10R and all its viral orthologs have only one cysteine pair that forms a CxxC motif and presumably donates electrons to FAD; the second cysteine pair is provided by the Cxx(x)C motif of A2.5L. Thus, the $E10R_{ss}A2.5L$ complex appears to be a functional analog of the single proteins DsbB, ERO1p, and ERV2p. The second protein in the cascade of disulfide bond formation invariably is a thioredoxin-like protein, namely DsbA in *E. coli*, PDI or its homologs in the yeast ER, and the G4L thioredoxin-like protein in poxviruses. Thus, the pathways of disulfide bond formation in such diverse systems as the *E. coli* periplasm, the yeast ER and the cytoplasm of vaccinia virus-infected cells appear to use the same general principles of thiol-disulfide transfer between protein components.

We thank Yan Xiang for helpful discussions and Gisella Storz and Chris Kaiser for useful comments on the manuscript.

1. Moss, B. (2001) in *Fields Virology*, ed. Howley, P. M. (Lippincott, Philadelphia), Vol. 2, pp. 2849–2883.
2. Locker, J. K. & Griffiths, G. (1999) *J. Cell. Biol.* **144,** 267–279.
3. Frand, A. R., Cuozzo, J. W. & Kaiser, C. A. (2000) *Trends Cell Biol.* **10,** 203–210.
4. Tu, B. P., Ho-Schleyer, S. C., Travers, K. J. & Weissman, J. S. (2000) *Science* **290,** 1571–1574.
5. Senkevich, T. G., White, C. L., Koonin, E. V. & Moss, B. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 12068–12073.
6. White, C. L., Senkevich, T. G. & Moss, B. (2002) *J. Virol.* **76,** 467–472.
7. Senkevich, T. G., Weisberg, A. & Moss, B. (2000) *Virology* **278,** 244–252.
8. Senkevich, T. G., Koonin, E. V., Bugert, J. J., Darai, G. & Moss, B. (1997) *Virology* **233,** 19–42.
9. Gerber, J., Muhlenhoff, U., Hofhaus, G., Lill, R. & Lisowsky, T. (2001) *J. Biol. Chem.* **276,** 23486–23491.
10. Lisowsky, T., Lee, J. E., Polimeno, L., Francavilla, A. & Hofhaus, G. (2001) *Dig. Liver Dis.* **33,** 173–180.
11. Benayoun, B., Esnard-Feve, A., Castella, S., Courty, Y. & Esnard, F. (2001) *J. Biol. Chem.* **276,** 13830–13837.
12. Lee, J., Hofhaus, G. & Lisowsky, T. (2000) *FEBS Lett.* **477,** 62–66.
13. Sevier, C. S., Cuozzo, J. W., Vala, A., Aslund, F. & Kaiser, C. A. (2001) *Nat. Cell Biol.* **3,** 874–882.
14. Hoober, K. L., Glynn, N. M., Burnside, J., Coppock, D. L. & Thorpe, C. (1999) *J. Biol. Chem.* **274,** 31759–31762.
15. Gross, E., Sevier, C. S., Vala, A., Kaiser, C. A. & Fass, D. (2002) *Nat. Struct. Biol.* **9,** 61–67.
16. Gvakharia, B. O., Koonin, E. & Mathews, C. K. (1996) *Virology* **226,** 408–411.
17. White, C. L., Weisberg, A. S. & Moss, B. (2000) *J. Virol.* **74,** 9175–9183.
18. McCraith, S., Holtzman, T., Moss, B. & Fields, S. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 4879–4884.
19. Weissman, J. S. & Kim, P. S. (1991) *Science* **253,** 1386–1393.
20. Debarbieux, L. & Beckwith, J. (1999) *Cell* **99,** 117–119.
21. Ritz, D. & Beckwith, J. (2001) *Annu. Rev. Microbiol.* **55,** 21–48.
22. Ravanello, M. P. & Hruby, D. E. (1994) *J. Virol.* **68,** 6401–6410.
23. Ravanello, M. P. & Hruby, D. E. (1994) *J. Gen. Virol.* **75,** 1479–1483.
24. Wolffe, E. J., Vijaya, S. & Moss, B. (1995) *Virology* **211,** 53–63.
25. Hooper, J. W., Custer, D. M., Schmaljohn, C. S. & Schmaljohn, A. L. (2000) *Virology* **266,** 329–339.
26. Frand, A. R. & Kaiser, C. A. (2000) *Mol. Biol. Cell* **11,** 2833–2843.
27. Jander, G., Martin, N. L. & Beckwith, J. (1994) *EMBO J.* **13,** 5121–5127.