Acidic C terminus of vaccinia virus DNA-binding protein interacts with ribonucleotide reductase

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ABSTRACT

Evidence from prokaryotic systems suggests that enzymes of dNTP synthesis are organized near the DNA replication apparatus, allowing direct utilization of dNTPs at their sites of synthesis. To investigate whether similar interactions exist within a eukaryotic environment, we have prepared anti-idiotypic antibodies to the small subunit of vaccinia virus ribonucleotide reductase, and we used these antibodies to search for proteins that interact with this enzyme. This approach identified a 34-kDa viral phosphoprotein, which, like ribonucleotide reductase itself, is localized within infected cells at DNA replication sites. After expression of its structural gene in Escherichia coli, the recombinant protein was purified and found (i) to bind tightly to single-stranded DNA and (ii) to stimulate enzymatic activity of vaccinia ribonucleotide reductase. These observations suggest a physical association between dNTP synthesis and DNA replication in this viral system.

To what extent is the enzymatic machinery for DNA precursor biosynthesis linked to the DNA replication apparatus? In prokaryotic cells, high replicative chain growth rates and low affinities of replicative DNA polymerases for dNTPs suggest functional connections between dNTP biosynthesis and utilization, which could help maintain high local concentrations of dNTPs at replication sites (1, 2). Considerable evidence, mostly from T4 phage-infected Escherichia coli, supports the existence of enzyme complexes that maintain such linkages. Evidence has been presented for similar complexes in eukaryotic cells (ref. 3; reviewed in ref. 2). Multi-enzyme aggregates have been described, but direct linkage in vivo between dNTP synthesis and DNA replication has not been demonstrated (2). Any work involving isolated enzyme aggregates suffers from possible artifacts involving disaggregation of weakly or transiently associated complexes or involving nonspecific aggregation, which might occur after artificial rupture of cells. Accordingly, we are using different approaches to determine whether dNTP synthesis is linked to DNA replication in a eukaryotic environment.

The system chosen is vaccinia virus-infected primate cells in culture. Vaccinia DNA replication occurs at cytoplasmic sites called virosomes. Therefore, viral DNA replication can be studied in some isolation from nuclear DNA metabolism of the host cell. Vaccinia virus encodes several enzymes of DNA metabolism, including ribonucleotide reductase, thymidine kinase, thymidylate kinase, dUTPase, DNA polymerase, and topoisomerase (4). rNPD reductase is of particular interest, because of its role in catalyzing the first reaction committed to DNA synthesis. Moreover, the structural genes for both large (R1) and small (R2) subunits of vaccinia rNPD reductase have been cloned and expressed in our laboratory, and both purified recombinant proteins are available in quantity (ref. 5; unpublished data). As in the mammalian or E. coli enzymes, both the R1 and R2 proteins are homodimers.

The present approach is analysis of anti-idiotypic antibodies (6). By generating an antibody to a ligand and then immunizing against those antibodies, some of the second-round antibodies might mimic the binding site of the ligand and, hence, bind to an unknown receptor. In principle, the technique is applicable to any protein-protein interactions, and it has revealed such interactions, both in our laboratory (7) and in others (cf. refs. 8 and 9). This paper describes the generation of anti-idiotypic antibodies directed against the small subunit of vaccinia virus rNPD reductase. Analysis of these antibodies has identified an interaction between viral rNPD reductase R2 protein and an abundant virus-encoded DNA-binding protein, which, like rNPD reductase itself, is localized at DNA replication sites.

MATERIALS AND METHODS

Antibodies. Polyclonal antisera against vaccinia rNPD reductase R1 and R2 proteins were prepared from fusion proteins containing the N-terminal portion of the E. coli TrpE repressor and the C-terminal portions of R1 α polypeptide and R2 β polypeptide, respectively (5). IgG fractions from each serum were prepared by chromatography on protein A-Sepharose (10). The anti-R2 IgG was used to generate anti-idiotypic antisera. IgG antibodies (1-3 mg) were injected into each of four New Zealand White rabbits, with booster injections at monthly intervals thereafter.

Intracellular Protein Localization by Immunofluorescence. BSC40 monkey kidney cells were grown in 12-well dishes on round coverslips until nearly confluent. Cells were infected with vaccinia virus strain WR at multiplicities of 1-5 plaque-forming units (pfu) per cell for 2-3 hr. For cells in which DNA replication sites were to be localized, bromodeoxyuridine (BrdUrd) labeling mixture (Amersham) at 1 μl/ml was added to the medium 20 min before harvesting. Cells were harvested, rinsed twice with phosphate-buffered saline (PBS), and fixed in 4% paraformaldehyde, followed by permeabilization with methanol. Serum diluted 1:20 in PBS containing 3% bovine serum albumin was added to the cells on each coverslip, and incubation was carried out for 30-60 min. Each sample was rinsed three times with PBS, followed by addition of biotinylated goat anti-rabbit IgG (Pierce) diluted 1:400. Incubation was carried out for 30 min, followed by three PBS rinses. Next, streptavidin/fluorescein isothiocyanate diluted 1:100 was added for 30 min, followed by washing and then addition of an undiluted monoclonal antibody to BrdUrd (Amersham). After 1 hr, the cells were again washed three times with PBS and then treated with rhodamine-conjugated goat anti-mouse IgG (Biomed) diluted 1:100. After three more PBS rinses, the coverslips were mounted in triethylene diamine (Sigma) on microscope slides and viewed in a Zeiss microscope with fluorescent optics and filters for fluorescein and rhodamine.

Immunoprecipitation Analysis. Confluent layers of BSC40 cells grown in Eagle's minimal essential medium (MEM) in

Abbreviation: pfu, plaque-forming units.

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60-mm dishes were infected with vaccinia virus at 15 pfu per cell. At the beginning of each labeling interval, the medium was replaced with 1.0 ml of methionine-free MEM plus [35S]methionine at 50 μCi/ml (1 Ci = 37 GBq), and incubation continued for 2 hr. In experiments in which a protein and its associated proteins were to be immunoprecipitated, BrdUrd labeling mixture (1 μl/ml) was added 20 min prior to harvest.

Next, the labeling medium was removed and the cells were washed twice with RIPA buffer (150 mM NaCl/1.0% Nonidet P-40/0.5% sodium deoxycholate/0.1% SDS/50 mM Tris-HCl, pH 8.0). Each cell preparation was then suspended in 800 μl of ice-cold RIPA buffer followed by brief sonication. In the time course experiment (Fig. 1A), 20 μl of antiserum was added to each extract, while the other experiments involved addition of either 5 μl of anti-anti-R2 or 100 μl of BrdUrd monoclonal antibody. Next, each preparation was incubated on ice for 1 hr. One hundred microliters of a 10% slurry of protein A-Sepharose (Sigma) in RIPA buffer was added, and each mixture was then rotated slowly for 1 hr at 4°C. The beads were centrifuged and washed extensively with PBS and prepared for SDS/polyacrylamide gel electrophoresis and fluorography.

Binding of Proteins in Virus-Infected Cell Extracts to Single-Stranded DNA-Cellulose. A confluent BSC40 cell culture in a 100-mm plate was infected with 20 pfu per cell in the presence of 30 μg of cytosine arabinonucleoside (araC). After 1 hr, the inoculum was replaced with [35S]methionine (20 μCi/ml) and 30 μg araC in MEM minus methionine, and infection continued for an additional 6 hr. The cells were washed twice with PBS and resuspended in buffer A containing 0.5 M NaCl (buffer A is 0.04 M Tris-HCl, pH 7.5/1 mM EDTA/10 mM MgCl2/2 mM CaCl2/1 mM 2-mercaptoethanol). Cells were sonicated, and the extract was incubated with DNase I (Sigma), dialyzed extensively against 0.1 M NaCl in buffer A, centrifuged at 27,000 × g for 15 min, and then loaded onto a 0.8-ml single-stranded DNA-cellulose column (Sigma). Elutions of 7 ml at 0.1 M, 5 ml at 0.3 M, 5 ml at 0.6 M, and 3 ml at 1 M, and 3 ml at 2 M NaCl were carried out at a flow rate of 4 ml/hr. One-milliliter fractions were collected and proteins were precipitated with 10% trichloroacetic acid.

Immunoprecipitation of 32P-Labeled Cells. Cells were grown to 95% confluency on 60-mm plates and infected with 15 pfu per cell in medium lacking F1. After 1 hr, the inoculum was removed and replaced with 1 ml of phosphate-free medium containing 10 μCi of [32P]. Two hours later, the cells were harvested and treated as in the [35S]methionine-labeling immunoprecipitations, except that instead of sonicating the extracts 50 μg of DNase I per ml was added only to the extracts immunoprecipitated with anti-anti-R2 antibody.

Isolating Agt11 Recombinant Expressing Anti-Anti-R2-Binding Domain. A Agt11 library created from a partial Hae III digest of WR vaccinia virus DNA was screened with anti-anti-R2 IgG diluted 1:1000, and positive plaques were picked and rescreened. One such recombinant phage was purified by CsCl gradient centrifugation and subjected to double-strand sequence analysis with Sequenase (United States Biochemical).

Cloning Vaccinia Open Reading Frame I-3 into pET11c. A clone of the HindIII I fragment in pBR322 was originally obtained from B. Moss (National Institutes of Health). A 1.5-kb fragment extending from the left-hand HindIII site to a BamHI site was subcloned into pT7-7 (11) to give pT7-7/H3-BH1-1.5. A fragment containing open reading frame I-3 was dropped out of this vector by digestion with Nde I and Rsa I, and this was ligated into pET11c (12) that had been linearized with BamHI, and the BamHI site was filled in with Klenow fragment of DNA polymerase and then cut with Nde I. The recombinant construct, pET11c/I-3, was transformed into E. coli strain BL21/DE3. The recombinant gene was expressed by growing 1 liter of cell culture at 30°C to A690 = 0.5 and inducing with 400 μM isopropyl β-D-thiogalactoside for 3 hr at 30°C.

Purification of Recombinant Viral DNA-Binding Protein. Cells from the above-described culture were resuspended in 3 ml of buffer A containing 0.5 M NaCl and lysed in a French pressure cell, followed by gentle rocking for 1 hr at 20°C. The lysate was dialyzed against low-salt buffer (0.1 M NaCl/0.02 M Tris-HCl, pH 7.5/1 mM EDTA), and then centrifuged at 27,000 × g for 10 min. The supernatant was loaded onto a 10-ml single-stranded DNA-agarose column (BRl) at a flow rate of 5 ml/hr. The column was washed first with 0.1 M NaCl and then with 0.5 M NaCl, both in 0.02 M Tris-HCl, pH 7.5/1 mM EDTA/1 mM 2-mercaptoethanol/10% (vol/vol) glycerol. Elution was continued until A690 dropped to baseline values for each salt concentration. The bulk of recombinant DNA-binding protein was eluted from the column with 2 M NaCl in the buffer described above.

RESULTS

Anti-Idiotypic Antibody to Viral rNDF Reductase Recognizes a 34-kDa Viral Protein. Polyclonal antiserum was generated against a fusion construct that expressed the 273 C-terminal residues of the 319-residue vaccinia β polypeptide, part of the rNDF reductase R2 protein. This antiserum recognized one viral protein, which has been identified as R2 (5). IgG was purified from this anti-R2 antiserum by chromatography on a protein A column. Two of four rabbits immunized against this IgG fraction yielded antibodies (anti-anti-R2) that reacted with one protein. This protein is viral in origin, as determined by both immunoprecipitation with radiolabeled viral proteins and immunoblot analysis. Fig. 1A shows immunoprecipitation analysis of [35S]methionine-labeled proteins produced after viral infection. Note that the one immunoreactive protein, of ≈ 34 kDa, is synthesized.
most rapidly early in infection, with labeling rates decreasing dramatically 4 hr postinfection.

We suggest that the 34-kDa polypeptide represents a protein with which vaccinia virus R2 protein interacts in vivo. Consistent with this interpretation, we found that precipitation of the 34-kDa protein by anti-anti-R2 antiserum was readily inhibited by purified recombinant R2 protein (Fig. 1B); this shows that antibodies reactive with the 34-kDa protein were derived from epitopes on the R2 portion, but not the TrpE portion, of the fusion protein used to generate the original R2 antiserum.

The 34-kDa Protein Is Localized to Viral DNA Replication Sites. If dNTP synthesis is linked to DNA replication, we would expect rNDP reductase to be localized to virosomes, where DNA replication occurs. Recent immunofluorescence analysis, which displays intracellular locations in situ, showed that much or most of the viral rNDP reductase R1 subunit is localized to sites of DNA replication (13).

Is the R2-reactive 34-kDa viral protein also localized in virosomes? To answer this question, we carried out similar immunofluorescence experiments with the anti-idiotype antiserum. In these experiments, we visualized sites of DNA replication by brief incorporation of BrdUrd before harvesting, followed by treatment of cells with a monoclonal antibody against BrdUrd (14). These experiments showed a discrete localization of the anti-idiotype antibodies to the same loci as the anti-BrdUrd antibody (Fig. 2). Note that unlabeled cells in the figure, presumably uninfected, show neither sites of extranuclear BrdUrd incorporation nor sites of anti-anti-R2 antibody deposition. Similar results were seen when uninfected cultures were analyzed, and control cultures that had not incorporated BrdUrd showed the same pattern of anti-anti-R2 antibody deposition as shown in Fig. 2 (results not shown). These results indicate that viral ribonucleotide reductase reductase interacts in vivo with a viral protein that is localized to DNA replication sites. In addition, these data support our earlier finding that ribonucleotide reductase itself is localized to these sites.

The 34-kDa Protein Is a DNA-Binding Protein. It is appealing to think that the R2-reactive 34-kDa protein plays a role in DNA replication, since it is localized to replication sites and its synthesis coincides with the period of most active DNA replication. Since many replication proteins bind to DNA, we asked whether the 34-kDa protein would precipitate along with BrdUrd-substituted DNA when the latter was treated with the anti-BrdUrd monoclonal antibody. As shown in Fig. 3A, this treatment did precipitate one radiolabeled protein, which comigrated with the R2-reactive 34-kDa protein. In cells that had not incorporated BrdUrd before har-

![Fig. 2. Immunocytochemical localization of protein reactive with R2 anti-idiotype antiserum. (A) Localization of protein reactive with anti-anti-R2 antiserum (green fluorescence). (B) Localization, in the same field, of DNA replication sites (red fluorescence).](image)

![Fig. 3. Anti-anti-R2 antiserum recognizes a DNA-binding phosphoprotein. (A) Precipitation by BrdUrd antibody. Infected cells were [35S]methionine-labeled from 1 to 3.5 hr postinfection. BrdUrd was added, as indicated, for the last 20 min of the labeling interval. Immunoprecipitation by preimmune serum (lane 1), anti-anti-R2 antiserum (lane 2), anti-BrdUrd antiserum (lane 4), anti-BrdUrd antiserum but cells not preexposed to BrdUrd (lane 5). Lane 3, molecular size markers (kDa). (B) Analysis of 32P-labeled proteins. Cells were labeled with 32P, from 1 to 3 hr postinfection. Lanes: 1, uninfected cells analyzed with anti-anti-R2 antiserum; 2, infected cells analyzed with anti-BrdUrd antiserum but without prior exposure to BrdUrd; 3, BrdUrd-treated infected cells analyzed with anti-BrdUrd antiserum; 4, infected cells analyzed with anti-anti-R2 antiserum.](image)
NaCl, DNAp34 is the only labeled protein in the eluate. The R2 protein itself, identified in separate immunoblotting experiments (data not shown), passed through the column in two peaks. Most was in the 0.1 M salt flow-through fractions, but 5–10% eluted sharply at 0.3 M NaCl, possibly because of its binding to DNAp34.

**Identification of the Structural Gene for DNAp34.** To characterize the 34-kDa protein, it was desirable to identify and express its structural gene. To this end, we screened a phage λgt11 expression library of *Hae* III restriction fragments of vaccinia virus DNA, using both anti-idiotypic and preimmune sera. DNA sequencing of a positive λ fusion clone revealed that the viral gene that encodes the 34-kDa protein is located in the *Hind* III I restriction fragment of the vaccinia virus genome. The open reading frame 1-3 lies immediately adjacent to open reading frame 1-4, the structural gene for the large subunit polypeptide (R1) of ribonucleotide reductase (16). The molecular mass predicted for open reading frame 1-3 is 30 kDa. Northern blot analysis indicated that the mRNA corresponding to this gene is present at both early and late times after infection (17).

The vaccinia virus insert in the immunoreactive λ clone that we sequenced contains DNA corresponding only to 81 C-terminal amino acids at the 3′ end. Therefore, an epitope to which the anti-idiotypic antibody corresponds must lie within the C-terminal domain of the DNAp34 protein, indicating that the interaction between rNTP reductase R2 protein and DNAp34 involves the C-terminal region of the latter protein.

**Purification and Characterization of Recombinant DNAp34.**

The open reading frame for DNAp34 was cloned into a pET11c vector expression system and overexpressed in *E. coli* strain BL21/DE3. A 34-kDa protein, immunoreactive with anti-anti-R2 antiserum, was synthesized in large quantities in these cells (∼1 mg/g wet weight of bacteria). We used a DNA-agarose column for large-scale purification of this protein. An abundant protein, identified as DNAp34, was eluted from the column at 2 M salt. Protein in this peak was ∼95% homogeneous, as estimated from a Coomassie blue-stained gel (Fig. 5).

This recombinant DNAp34 polypeptide has an apparent molecular mass on SDS gels of 34 kDa, and it is tightly bound to a single-stranded DNA-cellulose column (data not shown). Although 34-kDa protein begins to elute from this column at 0.6 M NaCl, the major elution peak is at 2 M NaCl and is much sharper than that seen in the earlier analysis of DNA-binding proteins in extracts of infected BSC40 cells. By contrast, the protein was completely eluted from a double-stranded DNA-cellulose column by 0.5 M NaCl, indicating specific affinity of the protein for single-stranded DNA (data not shown).

We used the purified recombinant DNAp34 protein to determine whether its interaction with R2 protein stimulates ribonucleotide reductase activity. As shown in Fig. 5, stimulation was evident. While the extent of stimulation was not large (∼1.5-fold), it was reproducible and specific. Stimulation was found in four independent experiments, while bovine serum albumin showed no stimulation of activity.

**DISCUSSION**

Anti-idiotypic antibodies present powerful reagents for analysis of protein–protein interactions. Using this approach, we discovered an interaction between two T4 phage-encoded dNTP synthetic enzymes (7). Application of the technique to vaccinia rNTP reductase was desirable, because in early experiments only ∼5% of the vaccinia R2 protein cosedimented with virosomes in centrifugal gradients (13). Did that 5% represent nonspecific association with virosomes, or did it represent a specific linkage that was disrupted by artificial lysis of cells? Our recent immunofluorescence data suggest...
that in vivo much or most of the ribonucleotide reductase is virosmo associated (13). Moreover, that association apparently involves an interaction between the reductase R2 protein and an abundant DNA-binding protein earlier identified as a major virosmal component. That protein has elsewhere been identified as polypeptide B (18–20), VDP 12 (21), and FP11 (22). It seems likely that all these terms identify the same protein and that that protein is identical to DNAP34, the protein shown here to interact with R2 protein of ribonucleotide reductase.

It is surprising that none of the four anti-anti-R2 antisera that we obtained contained antibodies directed against vaccinia R1 protein, given the structure of the viral ribonucleotide reductase as an R1–R2 complex. This may reflect an unusually weak interaction between R1 and R2. However, recall that the original R2 antibody was prepared against a fusion protein lacking the N-terminal region. If the missing sequence includes part of the R2 domain of interaction with R1, then anti-idiotypic antibodies to this domain would not be seen. Full-length R1 and R2 proteins are now available; anti-idiotypic antisera to both proteins remain to be developed.

Does DNAP34 play a role in DNA replication? While we do not know for certain, its location, its timing of synthesis, and its abundance suggest an affirmative answer. However, none of the existing replication-defective vaccinia mutants maps to this gene. We have attempted to block synthesis of DNAP34 by using an antisense vector directed against the translational start region for DNAP34. Infected cells carrying this vector showed 30–40% inhibition of DNA synthesis, suggesting that the protein does participate in replication (13). Interactions of this protein with viral DNA replication proteins are being studied in Paula Traktman’s laboratory (personal communication).

The affinity of DNAP34 for single-stranded DNA-cellulose is similar to that of well-characterized single-stranded DNA-binding proteins, such as bacteriophage T4 gene 32 protein, where a role in replication has been demonstrated (23). Adding to this circumstantial evidence is the fact that DNAP34 possesses an extremely acidic C-terminal domain, a general characteristic of this class of proteins. The predicted amino acid sequence (24) shows 17 aspartate and glutamate residues in the C-terminal 67 residues. A comparison of DNAP34 with T4 gp32, the phage single-stranded DNA-binding protein, shows that the two proteins share similar design features, even though they show little sequence homology. Both proteins have the potential to form four amphipathic helices (25), and both show extremely acidic C-terminal domains. T4 gp32 interacts with numerous proteins, and evidence suggests that the interactions, like those involving DNAP34, occur at the C terminus (25).

What is the relevance of these results to understanding the relationship between dNTP synthesis and cellular DNA replication in eukaryotes? Although our observations suggest that dNTPs are synthesized at or near replication sites in this viral system, one must be cautious in generalizing these results to cells. However, the stimulation of rNDP reductase by a DNA-binding protein is consistent with a physical linkage in this viral system, and it suggests an approach that might be informative if applied to eukaryotic cells.

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