Assembly of double-layered virus-like particles in mammalian cells by coexpression of human rotavirus VP2 and VP6

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Development in mammalian cells of a recombinant expression system that mimics the rotavirus capsid assembly process would be advantageous for studying the structural requirements for particle formation. To this end, we investigated the ability of a recombinant vaccinia virus system to produce double-layered virus-like particles. The genes coding for VP2 and VP6 proteins of the human rotavirus strain Wa were cloned and used to generate recombinant vaccinia viruses. Metabolic labelling of CV-1 cells infected with these recombinant viruses followed by immunoprecipitation with a polyclonal antiserum directed to Wa virus showed that VP2 and VP6 were efficiently expressed. The recombinant proteins were similar in size and immuno-reactivity to authentic rotavirus proteins. Biochemical and electron microscopy analyses demonstrated that simultaneous expression of VP2 and VP6 in mammalian cells resulted in the formation of intracellular spherical particles resembling double-layered rotavirus particles.

Rotaviruses, members of the family Reoviridae, are recognized as the principal viral cause of severe gastroenteritis in children and young animals. Their genome, which consists of 11 segments of double-stranded (ds) RNA, is enclosed in virus particles composed of three concentric protein layers (for a recent review see Mattion et al., 1994). The virus has an inner core consisting of the structural proteins VP1, VP2, VP3 and the dsRNA genome. This is surrounded by an intermediate shell of VP6 trimers and an outer layer composed of VP7 and spikes of dimeric forms of VP4 (Prasad et al., 1988, 1990). The structural proteins of rotavirus have been implicated in several roles during viral infection. VP2, the most abundant protein of the core, binds to viral RNA (Labbé et al., 1991) and may participate in the replication and encapsidation of the RNA genome. VP6 bears group- and subgroup-specific epitopes (Greenberg et al., 1983), and its removal from subviral particles has been associated with loss of viral transcriptase activity (Sandino et al., 1986), although VP6 by itself shows no polymerase activity. Both outer capsid proteins VP4 and VP7 induce neutralizing antibodies (Hoshino et al., 1985).

Gene cloning and expression have been successfully used to assess the structural and functional roles of individual viral proteins. In this regard, the in vivo assembly of virus-like particles after the simultaneous expression of their component proteins offers the possibility of studying protein interaction and viral morphogenesis. Furthermore, virus-like particles carrying relevant viral antigens may prove useful for the development of a vaccine.

It has been shown that recombinant rotavirus VP2 assembles into core-like particles (Labbé et al., 1991), and that coexpression of this protein with VP6 results in the formation of double-layered virus-like particles (Labbé et al., 1991; Tosser et al., 1992). Recently, triple-layered rotavirus-like particles were obtained in vivo after simultaneous expression of the capsid proteins VP2, VP4, VP6 and VP7 (Crawford et al., 1994). The in vitro assembly of purified recombinant VP4, VP6 and VP7 proteins into virus-like particles has also been reported (Redmond et al., 1993). All these communications described assembly reactions in insect cells using the baculovirus expression system. Therefore, as a first step toward studying the specific interactions of VP2 and VP6 in mammalian cells, we decided to investigate whether coexpression of these structural rotavirus proteins by recombinant vaccinia viruses results in the assembly of double-layered virus-like particles.

To produce full-length cDNAs of genes 2 and 6, genomic dsRNA purified from human Wa rotavirus-infected cells was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (M-MLV RT, Gibco BRL) and appropriate 3' specific antisense primers (see below) according to the manufacturer's directions. The cDNAs were amplified by PCR using the conditions described previously (González et al., 1993).
The amplification reactions contained the same 3'-specific antisense primers used in the RT reaction and appropriate 5'-specific sense primers. The primers were designed according to the published sequences of gene 2 (Ernst & Duhl, 1989) and gene 6 (Both et al., 1984) of the human Wa rotavirus strain. The sequences of the 5' sense and 3' antisense primers were as follows: gene 2, 5' GAAGTCCACATGGGCTACAGGAAG 3' (nucleotides 17-31) and 5' GGTGGATCCTTACAGTTGCAAT 3' (nucleotides 2689-2675); gene 6, 5' GTGTCGACATGGAGGTTCTGTAC 3' (nucleotides 24-38) and 5' GTCCGATCCCTCAGCTTCAACAT 3' (nucleotides 1217-1203). The sense and antisense primers also contain sequences that generated in the cDNA products SalI and BamHI restriction enzyme sites (underlined), respectively, to facilitate cloning. The PCR products were cloned into the vaccinia transfer vector pMJ601 (Davison & Moss, 1990) and completely sequenced by the dideoxynucleotide method (Sanger et al., 1977) to verify the absence of nucleotide misincorporations due to the amplification step. The genes coding for VP2 and VP6 were inserted into the vaccinia WR virus by homologous recombination between the plasmid transfer vector and vaccinia virus genomic DNA essentially as previously described (González et al., 1993). All recombinants were plaque-purified three times and identified by their ability to form blue plaques (coexpression of β-galactosidase) in the presence of the chromogenic substrate X-Gal and by hybridization to radiolabelled gene 2 and gene 6 DNA probes. The recombinant vaccinia viruses carrying the full-length genes 2 and 6 were designated vv-Wa-2 and vv-Wa-6, respectively.

To analyse the expression of VP2 and VP6, CV-1 cells were infected at high multiplicity (10 p.f.u. per cell) with either vv-Wa-2 or vv-Wa-6 and metabolically labelled at 4 h post-infection with [3H]leucine (200 μCi/ml, 151 Ci/mm; NEN) for 4 h in leucine-deficient DMEM containing 2% fetal calf serum. Cells were lysed in NP-40 lysis buffer in the presence of protease inhibitors (González & Burrone, 1991) and VP2 and VP6 were immunoprecipitated with guinea pig anti-Wa serum; this was followed by analysis on SDS–10% polyacrylamide gels and autoradiography enhanced by fluorography with En3Hance (NEN). Fig. 1(a) shows that CV-1 cells infected with vv-Wa-2 expressed a polypeptide of 92 kDa which comigrated with the VP2 protein found in rotavirus-infected cells (compare lanes 1 and 3).
addition to the 92 kDa polypeptide, two minor bands corresponding to polypeptides of apparent molecular mass 87000 and 82000 were detected (Fig. 1b). These bands have previously been characterized as VP2-related cleavage products for baculovirus-expressed bovine RF VP2 (Zeng et al., 1994). In the case of cells infected with vv-Wa-6, a polypeptide with the same molecular mass (41 kDa) as authentic VP6 was detected (compare lanes 1 and 4). Bands identified as recombinant rotavirus VP2 and VP6 proteins were absent from cell lysates infected with a vaccinia virus expressing the \( E. \) coli \( \text{lacZ} \) gene used as an experimental control (lane 2). Some cellular and/or vaccinia virus-encoded proteins which were non-specifically precipitated were observed in the immunoprecipitates of all cell lysates.

The quaternary structure of recombinant VP6 was investigated by immunoprecipitation with a subgroup II-specific monoclonal antibody followed by electrophoresis in the presence of reducing agent with and without boiling of the samples (Fig. 1c). When the immunoprecipitates of vv-Wa-6-infected cells were incubated at 37 °C, the monomeric form of VP6 observed in boiled samples (lane 1) disappeared and a product of high molecular mass was detected (lane 2). This band of about 140 kDa corresponds to the trimeric form of VP6 (Sabara et al., 1987). This result demonstrated that the expressed VP6 was in its native conformation.

To determine whether coexpression of the recombinant proteins resulted in double-layered particle formation, CV-1 cells coinfected with vv-Wa-2 and vv-Wa-6 were metabolically labelled with \([\text{H}]\)leucine for 4 h as described above. Cells were harvested and lysed in buffer containing 100 mM-Tris-HCl (pH 7-5), 0-1 mM-EDTA, 1% sodium deoxycholate, 1 mM-phenylmethylsulphonyl fluoride and 10 μg/ml aprotinin and clarified by centrifugation for 10 min at 12000 g. The resulting supernatant was ultracentrifuged through a 30% (w/v) sucrose cushion (González et al., 1993), and the pellet was immunoprecipitated and analysed by gel electrophoresis. Fig. 2(a) illustrates the presence of both recombinant VP2 and VP6 in the pelleted material. These proteins were not detected in the fraction that resulted from ultracentrifugation of the clarified cell culture medium of coinfected cells. Densitometric scanning of the autoradiograms of the particulate material obtained from cell lysates indicated that the bands corresponding to VP2 and VP6 had similar intensity. When the number of leucines present in each protein was taken into account (98 and 36 residues for VP2 and VP6, respectively), the ratio of VP6 to VP2 in the particles was 2-8. This value is somewhat lower than the stoichiometry expected (3-9) according to electron cryomicroscopy studies (Prasad et al., 1988, 1990). This difference may be due to contamination of the sucrose cushion pellet with cell debris.
bound VP2. Nevertheless, stoichiometry based on biochemical studies should be regarded as approximate estimates when compared to calculations derived from electron cryomicroscopy analyses (Crawford et al., 1994). The proportion of the intracellular VP2 and VP6 which assembled into particles was determined in three independent experiments. Under the experimental conditions described above, 25–30% and 42–55% of intracellular VP2 and VP6, respectively, assembled into particles. These values represent underestimates since they are probably affected by protein loss during particle purification.

Negative staining and electron microscopic analysis of the particulate fraction revealed that recombinant VP2 and VP6 proteins assembled into 60 nm spherical particles which were similar to double-layered virions (Fig. 2b). Recombinant particles consisted of a core surrounded by a thick protein layer. Double-layered rotavirus-like particles were never seen in cells infected with a control vaccinia virus.

In summary, we demonstrated in this report the suitability of the vaccinia virus expression system to produce intracellular recombinant double-layered virus-like particles in mammalian cells. The ability to form these particles by coinfection with recombinant vaccinia viruses expressing VP2 and VP6 should allow a more detailed analysis of the interactions between these viral proteins in the cytoplasm of mammalian cells, where the first stages of rotavirus morphogenesis take place. Site-directed mutagenesis of VP2 and VP6 genes followed by their coexpression may allow the mapping of the specific amino acids residues involved in intracellular targeting, assembly and interaction.

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