Expression of a Membrane-Anchored Glycoprotein, the Influenza Virus Hemagglutinin, by Dicistronic Replicons Derived from the Poliovirus Genome

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Mono- and dicistronic poliovirus replicons were constructed to express the influenza virus hemagglutinin, retaining its signal peptide and transmembrane region. Picornavirus genomes do not normally encode glycoproteins, and only the dicistronic replicon, in which the foreign and poliovirus sequences were separated by the encephalomyocarditis virus internal ribosomal entry site, replicated and expressed glycosylated hemagglutinin.

Poliovirus (PV), the prototype picornavirus, has a single-stranded RNA genome with positive-sense polarity. Direct translation of the genome is initiated at an internal ribosomal entry site (IRES) in the 5' noncoding region, resulting in a single polyprotein that is further processed into final products by autocatalytic cleavage by the viral 2A and 3C proteases. The PV genome has been engineered to express foreign proteins by inserting heterologous sequences in place of all or most of the capsid protein coding sequences, the P1 region of the genome. In this way, self-replicating RNAs or replicons that retain the ability to replicate for one cycle can be obtained by *in vitro* transcription of corresponding cDNAs (4, 10, 14). Upon transcapsidation into PV capsids, such PV replicons encoding foreign proteins were shown to induce immune responses against a variety of heterologous proteins when administered to mice transgenic for the PV receptor (3, 13). Recently, we demonstrated that naked-RNA immunization with PV-derived replicons can elicit humoral immune responses and constitutes an alternative strategy to DNA immunization (16). However, the use of such recombinant PV replicons in immunization strategies that would require the expression of surface glycoproteins is limited by the fact that it is not clear how the PV genome, which does not naturally encode glycoproteins, could be used as an expression vector for this purpose.

Doubts regarding the possibility of expressing glycoproteins from engineered PV replicons were first raised by Ansardi et al. (3), who found that the presence of a secretory pathway signal peptide and transmembrane anchor in the heterologous protein abrogated replication and that removal of the signal sequence restored this capacity. Likewise, Lu et al. (11) found that fusion of a heterologous protein containing a signal peptide to the P1 or to the P2-P3 PV precursor in the context of a full-length recombinant PV genome did not allow rescue of the corresponding virus. It is noteworthy that the signal sequence was located at the NH₂ terminus of the fusion polyproteins encoded by the recombinant genomes described in these two studies.

More recently, Anderson et al. observed that PV subgenomic replicons expressing the simian immunodeficiency virus (SIV) envelope SU protein, including its signal peptide, were replication competent when VP4 coding sequences were conserved in the replicon and the heterologous SU coding sequences were fused at their 3' end (1, 2). In these latter studies and according to previous results from the same authors (15), the SIV envelope protein was first synthesized as a cytosolic nonglycosylated VP4-SIV SU fusion protein; later, upon processing *in trans* by the 2A protease, the signal sequence of the SU protein became exposed at the NH₂ terminus, thus directing the SU protein to the endoplasmic reticulum. Supporting this scheme was the fact that only a portion of the SU protein expressed by the PV replicon was glycosylated in transfected cells. Interestingly, Dollenmaier et al. provided recent evidence that subgenomic replicons derived from another picornavirus, human rhinovirus 14, could express the glycosylated F protein of respiratory syncytial virus (8). However, since two similar constructs containing the genes for glycosylated secreted alkaline phosphatase and human growth hormone were unable to replicate, the authors concluded that the replication capacity of recombinant rhinovirus replicons depends on the specific foreign sequences inserted.

One hypothesis to explain the inhibition of replication by a signal peptide sequence inserted immediately following the PV polyprotein initiation codon could be that the signal peptide would send the nascent polyprotein or the whole viral polyome itself onto the rough endoplasmic reticulum and away from the site of RNA replication before the nonstructural proteins could be translated and released.

In order to explore this possibility, we constructed a dicistronic replicon (rΔP1-IR-HA) in which translation of the influenza virus hemagglutinin (HA) was uncoupled from that of the nonstructural P2-P3 PV sequences by the insertion of IRES sequences in between. Its ability to replicate and express the HA was then compared with that of a monocistronic replicon (rΔP1-E-HA) in which the HA sequences were fused in frame with the P2-P3 PV sequences.

Plasmid pΔP1-E-HA was derived from previously described plasmid pΔP1-E (16) containing the cDNA of the subgenomic PV replicon rΔP1-E from which the capsid protein coding sequences of P1 have been deleted and replaced with a

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FIG. 1. Schematic representation of plasmids encoding subgenomic recombinant mono- and dicistronic replicons derived from the PV genome. Plasmid pΔP1-E was derived from plasmid pT7-PV1-52, which contains the full-length infectious cDNA of PV type 1 Mahoney downstream of the T7 bacteriophage promoter (12). As already described (16), structural protein coding sequences (nt 746 to 3366) were replaced with a *SacI*, *XhoI*, *SalI* polylinker (not shown to scale) whereas sequences encoding the nine C-terminal amino acids of VP1 (VP1*) that comprise the optimal sequence for in-cis cleavage by the 2A protease were retained. Plasmid pΔP1-IR was constructed by insertion of sequences corresponding to the EMCV IRES at the *SalI* site of pΔP1-E, upstream of PV 2A sequences, in order to drive the translation of all of the P2 and P3 proteins required for replication while introducing stop codons (underlined; *TER*³) into all three reading frames downstream of the PV IRES and polylinker. The influenza virus HA coding sequences derived from influenza virus A/PR/8/34 (hatched box) were inserted behind the PV initiation codon of pΔP1-E and pΔP1-IR and either in frame with the remainder of the PV polyprotein sequences (pΔP1-E-HA) or upstream of the EMCV IRES sequences (rΔP1-IR-HA). Extra amino acid residues fused to the HA protein and derived from the PV or polylinker sequences are shown on either side in single-letter format, as well as extra amino acid residues at the N terminus of 2A^{pro} (black boxes). The tyrosine-glycine cleavage site of proteinase 2A^{pro} is indicated by an arrow. SP, HA signal peptide; TM, HA transmembrane sequence.

polylinker and a reconstituted 2A cleavage site sequence. The sequences coding for the HA, including a *SalI* site after the last codon of the open reading frame, were amplified by reverse transcriptase (RT) PCR from viral RNA of the mouse-adapted A/PR/8/34 (H1N1) influenza virus strain. The resulting DNA fragment was inserted between the Klenow-treated *SacI* and *SalI* sites of pΔP1-E, upstream of the reconstituted VP1-2A cleavage site and in frame with the remaining sequences encoding the PV polyprotein (Fig. 1). In addition, a silent mutation was introduced at codon 412 of the HA (GAA→GAG), which destroyed an *EcoRI* site in order to allow subsequent in

vitro transcription of the rΔP1-E-HA PV replicon from *EcoRI*-linearized plasmid.

Plasmid pΔP1-IR, containing the cDNA of a dicistronic subgenomic replicon, was constructed by insertion of the IRES sequences (nucleotides [nt] 306 to 845) from the encephalomyocarditis virus (EMCV) cDNA downstream of the *SalI* site of plasmid pΔP1-E and immediately upstream of the first codon of 2A so as to direct the translation of the P2-P3 region of the PV polyprotein. The IRES-containing fragment was amplified by PCR with primers designed to insert stop codons into all three reading frames before the EMCV IRES to ensure

termination of translation of sequences placed under the control of the upstream PV IRES (Fig. 1). In addition, the first four codons of the EMCV coding sequences (MATT) were conserved as a fusion to PV 2A sequences because they contribute to efficient initiation of translation of heterologous sequences by the EMCV IRES (5). Finally, influenza virus HA coding sequences were inserted, under the control of the PV IRES, between the Klenow-treated *SacI* and *SalI* sites of plasmid p Δ P1-IR in a fashion similar to that used for construction of plasmid p Δ P1-E-HA, yielding plasmid p Δ P1-IR-HA (Fig. 1).

To test the replication competency of the mono- and dicistronic PV replicons, synthetic recombinant RNAs, prepared by *in vitro* transcription with T7 RNA polymerase of the corresponding plasmids linearized with *EcoRI*, were transfected into HeLa cells by electroporation essentially as previously described (16). At different time intervals posttransfection, cytoplasmic RNA was extracted, slot blotted onto a nylon membrane, and analyzed by hybridization with a radiolabeled riboprobe complementary to PV RNA. No amplification of monocistronic r Δ P1-E-HA replicon RNA was observed (Fig. 2A, lane 2), while the parental r Δ P1-E replicon replicated efficiently (Fig. 2A, lane 1). On the other hand, both dicistronic replicons, r Δ P1-IR (lane 4) and r Δ P1-IR-HA (lane 5), were replication competent, although amplification of the corresponding RNAs was slightly delayed compared to that of the monocistronic r Δ P1-E replicon RNA. It should be noted that similar mono- and dicistronic recombinant replicons containing the sequences of the nonglycosylated influenza virus nucleoprotein (16) or *Aequorea victoria* green fluorescent protein (data not shown) were replication competent.

During completion of this study, Johansen et al. reported that dicistronic PV replicons may somehow exhibit genetic instability, the EMCV IRES being deleted upon multiple replication cycles, resulting in an in-frame fusion between the upstream heterologous gene and the downstream P2 PV sequences (9). To explore this possibility, RT-PCR analysis was performed on cytoplasmic RNA prepared from HeLa cells electroporated with r Δ P1-IR or r Δ P1-IR-HA replicon RNA (Fig. 2B). With primers +/523 and -/3542, designed to amplify the whole region of insertion and located, respectively, in the PV IRES and 2A sequences, no evidence of deletion of any of the influenza virus HA or EMCV IRES sequences could be found after genome replication (Fig. 2B). Similarly, no evidence of deletion was observed when we used primer +/62, spanning nt 62 to 83 at the 5' end of the PV genome, as a positive-sense primer (data not shown). In all cases, control reactions performed in the absence of AMV RT confirmed that the PCR products were not amplified from residual transcription template plasmid DNA (Fig. 2B).

It is not clear why the dicistronic genome constructed by Johansen et al. proved less stable than the r Δ P1-IR-HA replicon. It should nevertheless be noted that VP1-2A cleavage site sequences were not retained in the replicons described here, excluding the possibility of deletion of the EMCV IRES sequences alone or together with partial deletions in the HA sequences because these would result in expression of the 2A polypeptide as a fusion protein with additional N-terminal sequences. Such a 2A fusion protein is likely to be nonfunctional and could be translocated in the endoplasmic reticulum.

FIG. 2. Replication and stability of subgenomic PV replicons. (A) HeLa cells were mock transfected (lane 3) or transfected by electroporation with synthetic transcripts corresponding to monocistronic replicons r Δ P1-E (lane 1) and r Δ P1-E-HA (lane 2) or dicistronic replicons r Δ P1-IR (lane 4) and r Δ P1-IR-HA (lane 5). At the indicated times after transfection, cytoplasmic RNA was prepared, slot blotted onto a nylon membrane, and hybridized to a ³²P-labeled riboprobe complementary to nt 3417 to 4830 of the PV genome, essentially as previously described (16), prior to exposure on a Storm Phosphor-Imager (Molecular Dynamics). (B) HeLa cells were mock transfected or transfected by electroporation with synthetic transcripts corresponding to dicistronic replicons r Δ P1-IR and r Δ P1-IR-HA as indicated. Immediately after transfection (0) or 9 h later (9), total RNA was isolated with Trizol-LS reagent (Life Technologies) and reverse transcribed by AMV RT (Promega) with a negative-sense primer complementary to PV nt 3602 to 3624 (-/3602). These cDNAs and the corresponding plasmid DNAs were used as templates for PCRs with the Expand High Fidelity PCR System (Roche) and primers spanning PV nt 523 to 544 (+/523) and complementary to nt 3542 to 3564 (-/3542). The RT-PCR products were analyzed on a 1% agarose gel and visualized by ethidium bromide staining. The presence or absence of AMV RT in the reverse transcription reaction mixture is indicated by +RT or -RT, respectively. Lanes M contained molecular size markers (molecular sizes are indicated on the right in kilobases). In the schematic drawing of the r Δ P1-IR-HA RNA, solid arrows represent the primers used for RT-PCR, which are identified by numbers corresponding to their positions in the full-length PV genome. For definitions of abbreviations, see the legend to Fig. 1.

FIG. 3. Expression of the influenza virus HA by recombinant replicon rΔP1-IR-HA. HeLa cells were transfected by electroporation with replicon rΔP1-IR-HA (lane 3), rΔP1-IR (lane 4), rΔP1-E-HA (lane 5), or rΔP1-E (lane 6) or infected at a multiplicity of infection of 10 with influenza virus A/PR/8/34 (lane 1) or mock infected (lane 2). Cells were metabolically labeled with [35 S]methionine from 8 to 10 h after transfection and 12 to 18 h after infection. Cytoplasmic extracts were prepared at 10 h posttransfection or 18 h postinfection, and proteins were immunoprecipitated with polyclonal antibodies raised against influenza virus A/PR/8/34, analyzed by SDS-PAGE, and visualized by autoradiography. Molecular masses (kilodaltons) are shown on the right. The positions of the influenza virus HA, nucleoprotein (NP), and matrix protein (M1) from infected cells are indicated on the left.

It might, therefore, be anticipated that such deletion-containing replicons would replicate poorly, if at all, as functional 2A has been shown to be required for optimal replication of the PV genome.

Since the HA present in both the monocistronic and dicistronic constructs contained the signal peptide and transmembrane region, these results indicated that the uncoupling of translation between the heterologous glycoprotein and the PV nonstructural proteins restored the capacity to replicate and that the presence of the HA sequences directly following the PV starting codon seemed to abrogate the ability of a monocistronic construct to replicate, as previously reported by others for different glycoproteins (3, 11).

To exclude the possibility that this inability to replicate was due to a defect in polyprotein processing, *in vitro* translation of the RNA transcripts from the monocistronic (rΔP1-E-HA) and dicistronic (rΔP1-IR-HA) constructs was performed with rabbit reticulocyte lysates supplemented with HeLa S10 extracts and canine pancreas microsomal membranes. A typical sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) profile was observed for the PV nonstructural proteins (2A, 2C, 2BC, and 3CD) irrespective of the addition of microsomes. In addition, the influenza virus HA that was found to be glycosylated in the presence of microsomes could be detected (data not shown). Notably, the mature 2A protein

was detected for the rΔP1-E-HA transcripts, indicating that the reconstituted VP1*-2A cleavage site was correctly processed. This implies that since the inserted HA contains a transmembrane domain, its C terminus remained on the cytoplasmic side of the endoplasmic reticulum, allowing the VP1*-2A cleavage to release the nonstructural P2-P3 proteins in the cytoplasm, where replication was expected to take place.

Next, expression of the influenza virus HA was examined in HeLa cells transfected with the PV genome-derived rΔP1-E, rΔP1-E-HA, rΔP1-IR, and rΔP1-IR-HA RNAs or infected with influenza virus A/PR/8/34. Cells were pulse-labeled with [35 S]methionine for 2 h prior to being harvested at the time of peak expression, as determined in preliminary experiments, and cytoplasmic extracts were immunoprecipitated by using antibodies against the A/PR/8/34 virus. As shown in Fig. 3, dicistronic replicon rΔP1-IR-HA (lane 3) correctly expressed the HA molecule in a seemingly correctly glycosylated form, as it comigrated with the HA produced in cells infected with influenza virus (lane 1). No immunoreactive proteins could be detected from cells transfected with the nonreplicating rΔP1-E-HA RNA (lane 5) or from cells transfected with RNA derived from empty vectors rΔP1-E and rΔP1-IR.

To further characterize the immunoreactive protein expressed by the rΔP1-IR-HA replicon, enzymatic deglycosylation of proteins was performed. Metabolically labeled cytoplasmic extracts were prepared from HeLa cells transfected with the rΔP1-IR-HA replicon or as a control, infected with influenza virus A/PR/8/34, and immunoprecipitated with anti-A/PR/8/34 antibodies as described above. The bound proteins were eluted from the protein A-Sepharose in denaturing buffer (50 mM sodium phosphate [pH 7.5], 0.5% SDS, 1% β -mercaptoethanol), deglycosylated by incubation with 250 U of peptide N-glycosidase F (PNGase F) in denaturing buffer

FIG. 4. Glycosylation analysis of the HA molecule expressed by recombinant dicistronic replicon rΔP1-IR-HA. HeLa cells were transfected by electroporation with rΔP1-IR or rΔP1-IR-HA replicon RNA or infected with influenza virus and, at peak expression, metabolically labeled with [35 S]methionine as described in the legend to Fig. 3. Cytoplasmic extracts were prepared at 10 h posttransfection or 18 h postinfection, and proteins were immunoprecipitated with polyclonal antibodies raised against influenza virus A/PR/8/34. They were then subjected to PNGase F treatment (+) or mock treated (-), analyzed by SDS-PAGE, and visualized by autoradiography. All samples were run on the same gel, but the left half was exposed for a longer time for clarity. Molecular masses (kilodaltons) are shown on the right. The positions of the influenza virus HA, nucleoprotein (NP), and matrix protein (M1) from infected cells are indicated on the left.

FIG. 5. Expression of the HA molecule at the surface of cells transfected with recombinant dicistronic replicon rΔP1-IR-HA. HeLa cells were mock transfected (dotted line) or transfected by electroporation with rΔP1-IR (plain line) or rΔP1-IR-HA (bold line) replicon RNA. At 8 h posttransfection, cells were detached from the plates with PBS-EDTA and HA expression was determined by labeling of the cells with rabbit anti-A/PR/8/34 polyclonal antibodies. Bound antibodies were detected with fluorescein isothiocyanate-labeled anti-rabbit immunoglobulin G antibodies, and after fixation of the cells in PBS-paraformaldehyde, flow cytometry analysis was performed on a FACScalibur fluorospectrometer (Becton Dickinson).

with 1% NP-40 for 60 min at 37°C, and analyzed by SDS-PAGE (Fig. 4). After enzyme treatment, the same decrease in apparent molecular mass was observed in HA-related proteins expressed from both replicon-transfected (lane 6) and virus-infected (lane 4) cells. This confirmed that the HA molecule produced by rΔP1-IR-HA was indeed glycosylated to the same extent as the native HA.

Whether the HA expressed by this recombinant dicistronic replicon was present at the cell surface was examined by fluorescence-activated cell sorter analysis of HeLa cells transfected with the dicistronic rΔP1-IR and rΔP1-IR-HA replicons. HeLa cells were detached from the plates with phosphate-buffered saline (PBS) supplemented with 2 mM EDTA at 7 h posttransfection, and HA expression at the cell surface was revealed by labeling with primary rabbit anti-A/PR/8/34 polyclonal antibodies and secondary fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G antibodies. Flow cytometry analysis revealed that more than 50% of the cells expressed the HA molecule on the cell surface (Fig. 5). Thus, despite the fact that the PV 2B and 3A polypeptides have been shown to alter protein traffic through the host secretory pathway (6, 7), this effect appeared not to be strong enough to block the surface expression of a replicon-encoded glycoprotein.

Our findings thus demonstrated that it is possible to express a glycosylated protein at the cell surface by using recombinant subgenomic RNAs derived from the PV genome that retain replication competency. This required, however, that expression of the heterologous protein and of the PV proteins be

uncoupled by means of insertion of an IRES sequence. They further suggest that the presence of a signal peptide at the immediate NH₂ terminus of the polypeptide may send, during translation, the entire HA-P2-P3 polypeptide encoded by the monocistronic PV replicon rΔP1-E-HA to the rough endoplasmic reticulum before the 2A cleavage site could function to liberate the HA glycoprotein from the remaining P2-P3 polypeptide. As a result, in the case of the monocistronic replicon, the PV nonstructural proteins would be liberated in the cytoplasm but not in the vicinity of the clusters of neosynthesized vesicles where PV replication complexes are formed. In the case of dicistronic PV replicon rΔP1-IR-HA, this would obviously not occur since the signal peptide-containing HA sequences are not fused to the PV nonstructural P2-P3 polypeptide. Our findings also imply that the presence of a signal peptide sequence in a PV replicon cannot, by itself, sequester the whole viral polysome close to the rough endoplasmic reticulum and away from the site of PV replication as previously suggested (11).

In previous studies, induction of immune responses against the encoded foreign antigen has been demonstrated after immunization of mice with monocistronic PV replicons whether encapsidated for PV receptor-transgenic animals (3, 13) or as naked RNA for normal mice (16). Since many neutralization determinants of infectious agents are located on surface glycoproteins, the results reported here will allow the rational design of dicistronic replicons based on the genome of PV or other picornaviruses and the determination of their usefulness for induction of protective immune responses in suitable animal models.

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