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Expression of soluble forms of rubella virus glycoproteins in mammalian cells

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Abstract

Rubella virus (RV) virions contain two envelope glycoproteins, E1 and E2. Removal of hydrophobic regions in their carboxyl termini by genetic engineering caused them to be secreted rather than maintained in cell membranes of transfected COS cells. Truncated E2 was secreted in the absence of E1, whereas E1 lacking its transmembrane domain required coexpression of E2 for export from the cell. Secreted E2 was found to contain both O-linked and N-linked complex glycans, whereas secreted E1 retained virus neutralization and hemagglutination epitopes, suggesting the possibility of using soluble RV antigens as subunit vaccines and for serodiagnostic purposes. Stable Chinese hamster ovary cell lines secreting RV E1 were constructed for large scale preparation of recombinant E1.

Key words: Rubella virus; Expression; Secretion; Soluble protein

1. Introduction

Rubella virus (RV) is an important human pathogen, since first trimester infection frequently results in various birth defects collectively known as congenital rubella syndrome (Cooper and Buimovici-Klein, 1985). Currently, vaccination with live attenuated virus is the prophylactic measure of choice. However, complications such as polyarthralgia and arthritis can result, although rarely, from such immunization.

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The RV virion consists of a 40S single-stranded polyadenylated RNA encapsidated by a capsid protein which is in turn enclosed in an envelope consisting of two virus encoded glycoproteins E1 and E2 (Oker-Blom et al., 1983). The structural proteins of RV are initially translated as a precursor polyprotein in the order NH_2-C-E2-E1-COOH from a 24S subgenomic mRNA (Oker-Blom, 1984). After synthesis, the polyprotein is cleaved to yield two membrane glycoproteins, E1 (58 kDa) and E2 (42–47 kDa), and a basic 33 kDa capsid (C) protein (Oker-Blom, 1984; Oker-Blom et al., 1983). E1 and E2 comprise the spike complex and are thought to exist as heterodimers (Waxham and Wolinsky, 1983; Hobman et al., 1993). Insertion of glycoproteins E1 and E2 into the endoplasmic reticulum (ER) is mediated by two independently functioning signal peptides which are cleaved by host signal peptidase (Hobman et al., 1988; Hobman and Gillam, 1989). RV glycoproteins form a heterodimer in the ER before they are transported to and retained in the Golgi complex (Hobman et al., 1993; Baron and Forsell, 1991). In transfected COS cells, small quantities of E2 can be detected at the cell surface in the presence or absence of E1 (Hobman and Gillam, 1989; Hobman et al., 1990). E2 from RV virions has been shown to contain sialylated O-linked carbohydrates (Lundstrom et al., 1991; Sanchez and Frey, 1991).

Since antibodies with viral neutralizing (VN) and hemagglutination inhibiting (HAI) activities are directed against E1 (Waxham and Wolinsky, 1985; Green and Dorsett, 1986), it has been suggested that E1 is the major target of the B-cell response. Therefore, a candidate subunit vaccine would contain E1 in an immunogenic form. The biological role of E2 is not well defined, although it has been reported to possess strain-specific epitopes (Dorsett et al., 1985) and possibly at least one neutralizing domain (Green and Dorsett, 1986).

RV E1 and E2 are rich in cysteine residues and contain intramolecular disulfide bridges that are important in maintaining proper protein folding (Qiu et al., 1992a, 1992b). RV glycoproteins purified from virions show greatly reduced antigenicity compared with intact virus (Loo et al., 1986). This is presumably due to disruption of the conformation of E2 and E1 during isolation. Bio-engineered E1 expressed in *Escherichia coli* is low in immunogenicity as compared to the native E1 and does not elicit the production of HAI antibodies, although it is able to induce a low level of VN antibodies (Terry et al., 1989). Expression of soluble forms of RV E1 and E2 proteins in cultured cells would facilitate isolation of antigenic E1 and E2 proteins for biochemical studies as well as development of serodiagnostic assays and subunit vaccines for RV. In this study, we have investigated the possibility of expressing soluble forms of RV E1 and E2 proteins in cultured cells that can be used for the large-scale preparation of immunogenic E1 and E2.

2. Materials and methods

2.1. Plasmid construction

RV cDNAs encoding various combinations of the structural proteins are shown in Fig. 1. All cDNAs were subcloned between the *Eco*RI and *Hin*dIII sites of the

expression vector pCMV5 (Andersson et al., 1989) downstream from the human cytomegalovirus immediate early gene promoter. The construction of pCMV5-E1 (Fig. 1a) and pCMV5-E1E2 (Fig. 1e) has been previously described (Hobman et al., 1988; Hobman and Gillam 1989). The cDNA (E1ΔTM) was created by removal of 27 amino acid residues encoding the transmembrane anchor domain of E1 using site-directed mutagenesis (Kunkel, 1985) with the oligonucleotide pGTAGTA-CAAGCATTTCTCCGCCCAGGTCT. This oligonucleotide is complementary to sequences flanking the transmembrane domain of E1 but not to the transmembrane region itself. E1TMCT$^-$ (Fig. 1c) was constructed by changing the amino acid residue at position 441 from GAG (Glu) to TAG (Stop) using the mutagenic oligonucleotide, pAGCCCACT*A*CGCCCAGG (the mutated base is underlined). The construct, E1Δ*Bam*HI (Fig. 1d) was made by deleting the *Bam*HI and *Hin*dIII fragment of E1 (Fig. 1a). The ends of the plasmid were repaired with the Klenow enzyme and ligated in the presence of an *Xba*I linker (pC*TAG* TC *TAG* AG *TAG*) with three stop codons in different reading frames. The truncated E2 cDNA, E2ΔTM (Fig. 1g) was constructed by using convenient restriction sites in the E2 cDNA. The RV cDNA from pCMV5-E2 (Hobman et al., 1990) was first subcloned into the *Eco*RI and *Hin*dIII sites of the plasmid pSPT19 (Pharmacia). To remove the putative transmembrane domain of E2, the region encoding the C-terminal 60 amino acids was deleted by cutting the plasmid with *Xba*I followed by partial digestion with *Nco*I. The *Xba*I and *Nco*I termini were made blunt with T4 DNA polymerase and ligated using T4 DNA ligase. E1E2ΔTM (Fig. 1f) was created by replacing the *Bam*HI-*Hin*dIII fragment from pCMV5-E2E1 (Fig. 1e) with the *Bam*HI-*Hin*dIII fragment from E1ΔTM (Fig. 1b), resulting in the plasmid pCMV5-E2E1ΔTM.

2.2. Transfection, metabolic labelling, immunoprecipitation and endoglycosidase digestion

COS cells were transfected with recombinant plasmids and labelled with [^{35}S] methionine. RV-specific proteins were immunoprecipitated as previously described (Hobman and Gillam, 1989). The immune complexes were washed and the RV antigen was eluted into 100 mM sodium citrate (pH 5.5), 0.15% SDS, 1 mM phenylmethylsulfonyl fluoride at 100°C for 5 min and analyzed by SDS-PAGE (Laemmli, 1970). Some immunoprecipitates were digested with endoglycosidase H (5 mU/50 μl), N-glycosidase F/endoglycosidase F (200 mU/50 μl), O-glycosidase (2 mU/50 μl), or neuraminidase (10 mU/50 μl) as described by the supplier (Boehringer Mannheim).

2.3. Generation of CHO cell lines secreting RV E1 antigen

Using Lipofectin (Gibco), we cotransfected dihydrofolate reductase deficient (dhfr$^-$) Chinese hamster ovary cells (CHODG44) (Urlaub and Chasin, 1980) with pCMV5-E2E1 ΔTM and the plasmid pFR400 (Simonsen and Levinson, 1983). The

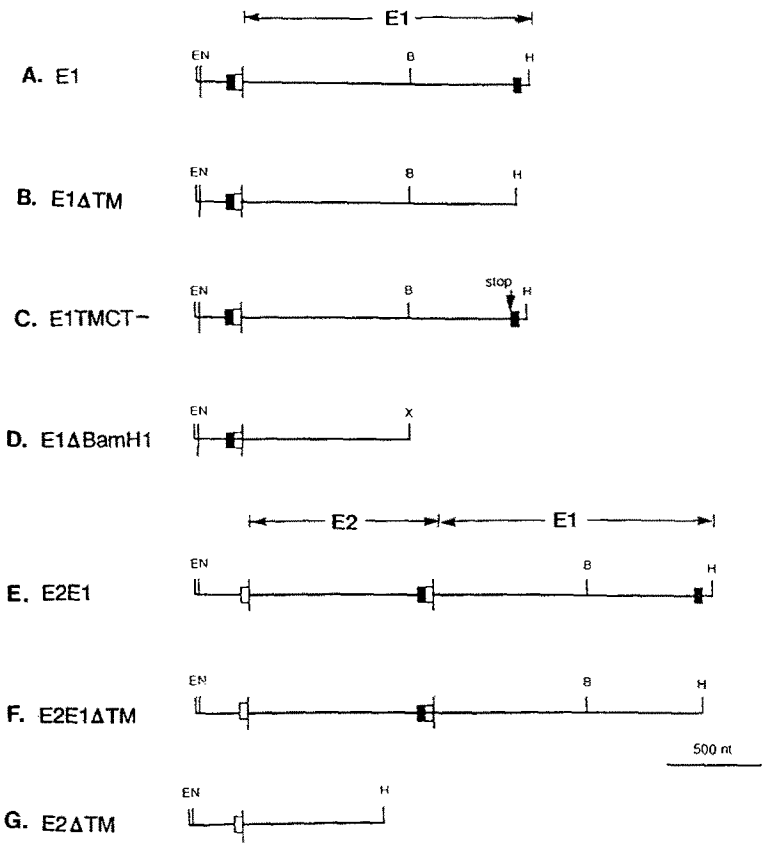

Fig. 1. Diagram of RV cDNAs. Respective portions of E2 and E1 genes are indicated above constructs. Intergene borders are marked by vertical lines extending through the cDNAs. The translation start site of the capsid protein is utilized in all constructs. Signal peptides are indicated by open boxes and putative transmembrane regions are shown by solid boxes. Restriction endonuclease sites are shown as E = *Eco*RI, H = *Hin*dIII, B = *Bam*HI, and N = *Nco*I, X = *Xba*I. RV cDNAs were subcloned between the *Eco*RI and *Hin*dIII sites of pCMV5 (Andersson et al., 1989). (a) E1 cDNA; (b) E1ΔTM cDNA; (c) E1TMCT$^-$ cDNA; (d) E1Δ*Bam*HI cDNA; (e) E2E1 cDNA; (f) E2E1ΔTM cDNA; (g) E2ΔTM cDNA. The bar represents approximately 500 nucleotides.

latter plasmid contains a mouse dhfr gene under the control of the SV40 early promoter which can be used as a selectable marker. Clones were selected for their ability to grow in dialyzed fetal bovine serum (which does not contain nucleosides), indicating that they were expressing the transfected dhfr gene. Selected clones were expanded and screened for the presence of RV antigen by indirected immunofluorescence (Hobman et al., 1990) and secretion of RV E1 by radioimmunoprecipitation.

3. Results

3.1. Expression of truncated E1 mutants

Sequence analysis and in vitro translocation experiments suggest that RV E1 is a type 1 membrane glycoprotein anchored in the lipid bilayer by a C-terminal hydrophobic domain (Clarke et al., 1987; Hobman et al., 1988). Removal of this domain (TM) should convert it into a soluble protein. Codons for a 27-amino acid peptide encoding the transmembrane anchor of E1, located 13 residues from the C-terminus of the protein (Fig. 1a), were deleted by using oligonucleotide-directed mutagenesis (Kunkel, 1985). The resultant E1ΔTM cDNA was cloned into the *Eco*RI and *Hin*dIII sites of the eukaryotic expression vector pCMV5 (Andersson et al., 1989) and transfected into COS cells using DEAE-dextran (500 μg/ml) as previously described (Hobman et al., 1988). Forty hours post-transfection, cells were pulse-labelled with 100 μCi [^{35}S]-methionine for 30 min and chased for the indicated times in medium with excess unlabelled methionine. Cells were lysed and immunoprecipitates of RV proteins were prepared using human anti-RV serum as described (Hobman et al., 1989). Samples were subjected to SDS-PAGE on 10% polyacrylamide gels followed by autoradiography. The E1ΔTM glycoprotein was not detected in the medium of transfected cells after a 3-h chase period, nor was there any evidence of N-linked sugar processing (Fig. 2a). E1ΔTM glycoprotein remained completely sensitive to endo H even after a 6-h chase period (not shown) suggesting that transport was arrested before the medial Golgi apparatus. Moreover, indirect immunofluorescence indicated that E1ΔTM protein was present largely in the rough ER (not shown) in contrast to wild type E1, which accumulates in a post-rough ER, pre-Golgi compartment (Hobman et al., 1990; 1992).

RV E1 may contain a signal that blocks its transport, as has been described for a limited number of other membrane glycoproteins (Colley et al., 1989; Jackson et al., 1990; Machamer and Rose, 1987; Nilsson et al., 1989). Domains known to date that cause retention of membrane proteins in the ER or Golgi apparatus are located within membrane-spanning peptides or in the immediate proximity. To determine if this were the case with E1, we made the construct, E1Δ*Bam*HI (Fig. 1d) that encodes an E1 protein lacking the C-terminal 173 amino acids including the putative transmembrane region and flanking luminal and cytoplasmic domains. COS cells were transfected with pCMV5-E1Δ*Bam*HI and labelled with [^{35}S] methionine. Following a 3-h chase period, none of the E1Δ*Bam*HI glycoprotein could be detected in the medium of radiolabelled transfected cells (Fig. 2a). Consistent with these observations, the 39-kDa deletion mutant protein was distributed in a cytoplasmic reticular pattern characteristic of the ER (not shown). For unknown reasons, we consistently observed that E1Δ*Bam*HI glycoprotein was expressed at lower levels per cell than E1ΔTM and wild type E1 proteins (not shown). One problem associated with identifying retention signals by deletion analysis is that very often the resultant proteins do not fold properly and are retained in the ER. In order to address the question of whether the cytoplasmic tail of E1 was preventing E1ΔTM protein from being secreted, we created

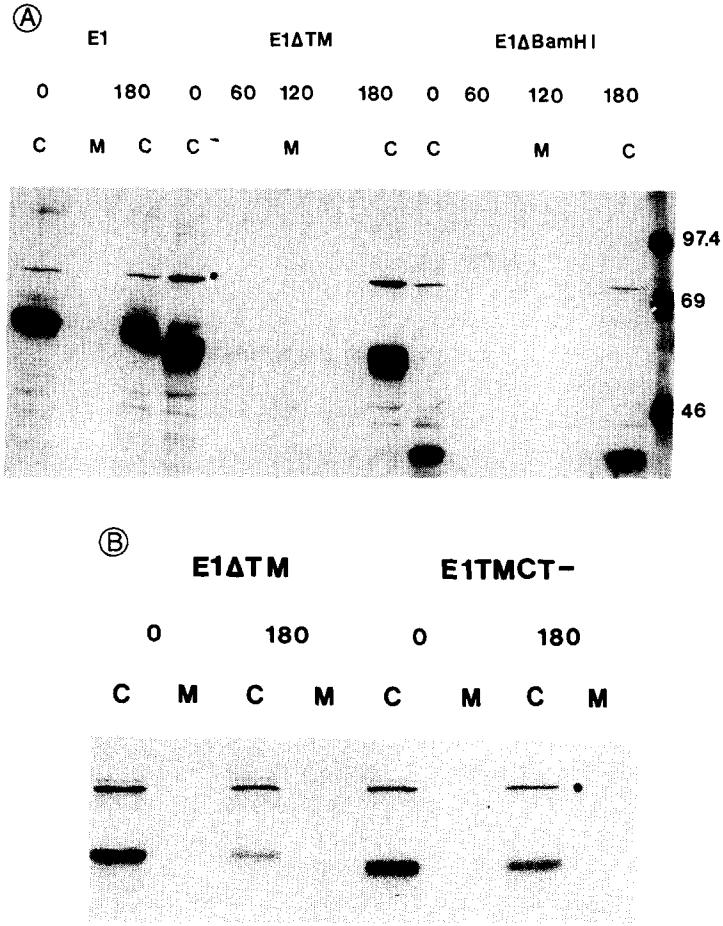

Fig. 2. Expression of E1ΔTM, E1Δ*Bam*HI and wild type E1 in COS cells. Radiolabelled protein standards (kDa) were included for reference. (C) and (M) indicate immunoprecipitates made from cell lysates and medium respectively. Only 25% of the total immunoprecipitate from cell lysates was loaded, whereas the whole of the media samples were loaded. The ER protein BiP is indicated with a dot. (a) Autoradiogram showing that E1, E1ΔTM, and E1Δ*Bam*HI glycoproteins are retained in COS cells. (b) Expression of E1TMCT$^-$ and E1ΔTM. Transfected COS cells were pulse-labelled for 30 min with [^{35}S]-methionine and chased for 0 and 180 min before cell lysates and medium samples were immunoprecipitated. 25% of each cell lysate sample was loaded. E1ΔTM glycoprotein was run on this gel to show the relative size of E1TMCT$^-$.

E1TMCT$^-$ (Fig. 1c). The codon Glu 441, immediately preceding the 27-amino acid transmembrane domain of E1, was changed from GAG to TAG, such that translation of the anchor and cytoplasmic domains did not occur. We found that the E1TMCT$^-$ glycoprotein behaved similarly to E1ΔTM and E1Δ*Bam*HI proteins in that it remained cell-associated in transfected COS cells, and was not detected in the medium after a three hour chase period (Fig. 2b).

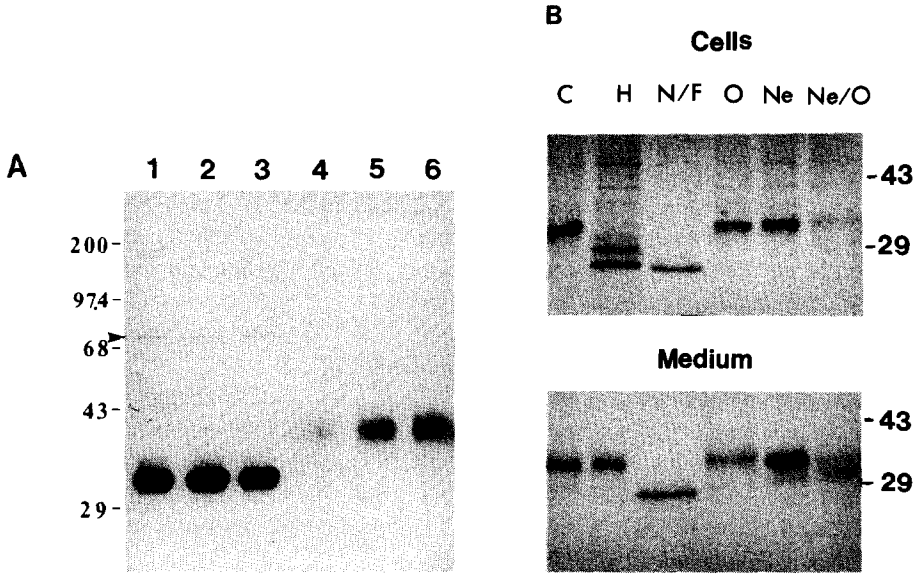

Fig. 3. Expression of E2ΔTM in COS cells. (a) Secretion of E2ΔTM. Cells expressing pCMV5-E1ΔTM were pulse-labelled with [^{35}S]methionine as above and chased for one, two and three hours as indicated. Note: 5% of the total cellular immunoprecipitate was loaded onto the gels compared to the whole of the RV proteins recovered from the medium. Lanes, 1, 2, and 3 represent cellular immunoprecipitates after one, two and three hours of chase respectively, and lanes 4, 5, and 6 represent the corresponding secreted proteins. The protein marked by an arrowhead was previously shown to be BiP (Hobman et al., 1990). Mobilities of protein standards are indicated (kDa). (b) Secreted E2ΔTM contains both N-linked and O-linked glycans. COS cells transfected with pCMV5-E2ΔTM were pulsed for 30 min with [^{35}S]-methionine and chased for 6 h. Immunoprecipitates were digested with endoglycosidase H (H), N-glycosidase F/endoglycosidase F (N/F), O-glycosidase (O), neuramidase (Ne), or neuramidase/O-glycosidase (Ne/O). Cells = immunoprecipitates from cell lysates; medium = immunoprecipitates from chase medium. The control in the absence of endoglycosidases is indicated (C). Mobilities of protein standards are indicated (kDa).

3.2. Expression of anchorless E2

To determine if truncated E2 glycoprotein could be secreted from COS cells, a truncated E2 cDNA, E2ΔTM (Fig. 1g), was constructed by using convenient restriction sites in the E2 cDNA. The E2ΔTM cDNA was then subcloned into pCMV5 and transfected into COS cells, which were used for biosynthetic labelling experiments. We found that the E2ΔTM glycoprotein (37 kDa) was secreted in a time-dependent manner (Fig. 3a) and contained endo H-resistant sugars (Fig. 3b, medium, lane H). In contrast, the 32 kDa intracellular form of E2ΔTM contained mainly endo H-sensitive sugars (Fig. 3b, cells, lane H). Digestion with the enzyme mixture (N-glycosidase F/endoglycosidase F) that hydrolyzes N-linked glycans of the high mannose and the complex type, reduced the size of intracellular (32 kDa) and secreted (37 kDa) E2 to 24 and 28 kDa respectively, indicating the presence of high mannose and complex type N-linked sugars on these proteins (Fig. 3b, lane

N/F). To investigate the possibility that the higher apparent molecular weight observed in the secreted E2 was due to the presence of O-linked sugars, immunoprecipitates were digested with O-glycosidase before and after neuraminidase treatment. The mobility of the intracellular E2 was not affected by the treatments (Fig. 3b, cells), whereas a lower molecular weight band was observed in the secreted E2 after the treatment with neuraminidase and O-glycosidase (Fig. 3b, medium), demonstrating the presence of O-linked glycans on the secreted form of E2. The slight decrease in the apparent molecular weight of the secreted E2 after neuraminidase treatment indicated that some of the oligosaccharides contained terminal sialic acid (Fig. 3b, medium, lane Ne).

3.3. Secretion of anchorless E1 requires coexpression of E2

Previously it was demonstrated that coexpression of E2 results in transport of a limited amount of E1 to the surface of COS cells (Hobman et al., 1990). To determine if E1ΔTM protein would be secreted when coexpressed with E2, a cDNA encoding the RV E2 gene and E1ΔTM protein was created by replacing the *Bam*HI-*Hin*dIII fragment from the pCMV5-E2E1 (Fig. 1e) (Hobman and Gillam, 1989) with the *Bam*HI-*Hin*dIII fragment from pCMV5-E1ΔTM (Fig. 1b), resulting in the plasmid pCMV5-E2E1ΔTM (Fig. 1f). Following transfection of this plasmid into COS cells, a 54 kDa soluble E1 glycoprotein could be detected in the medium after 60 min of chase (Fig. 4a), suggesting that the 27-amino acid peptide deleted from E1 was indeed the only membrane-spanning region of this protein. RV E2 was also present in the medium of these cells, although in much lower amounts (Fig. 4a). At present it is unclear whether proteolytic cleavage occurred to release E2 into the medium. Alternatively, the interaction of E2 with E1ΔTM may have altered its conformation such that it was not as stable in the membrane. In contrast, no E1 was detected in the medium of cells expressing proteins from pCMV5-E1ΔTM alone or from pCMV5-E2E1 after four hours of chase, although some E2 could be detected in the medium in the latter case (Fig. 4b, lane 4, arrowhead).

3.4. Expression of secreted E1 in transfected CHO cells

In order to be useful for the production of soluble E1 for subunit vaccines or for diagnostic purposes, a better expression system than transient transfection is necessary. Using Lipofectin (Gibco) we cotransfected dhfr$^-$ Chinese hamster ovary cells (CHO DG44 [Urlaub and Chasin, 1980]) with pCMV5-E2E1ΔTM and the plasmid pFR400 (Simonsen and Levinson, 1983). The latter plasmid contains a mouse dhfr gene under the control of the SV40 early promoter, which can be used as a selectable marker. Clones were selected for their ability to grow in dialyzed fetal bovine serum (i.e., in the absence of nucleosides), indicating that they were expressing the transfected dhfr gene. Selected clones were expanded and screened for the presence of RV antigen by indirect immunofluorescence and secretion of RV E1 by radioimmunoprecipitation. Fig. 5 shows the results of a biosynthetic

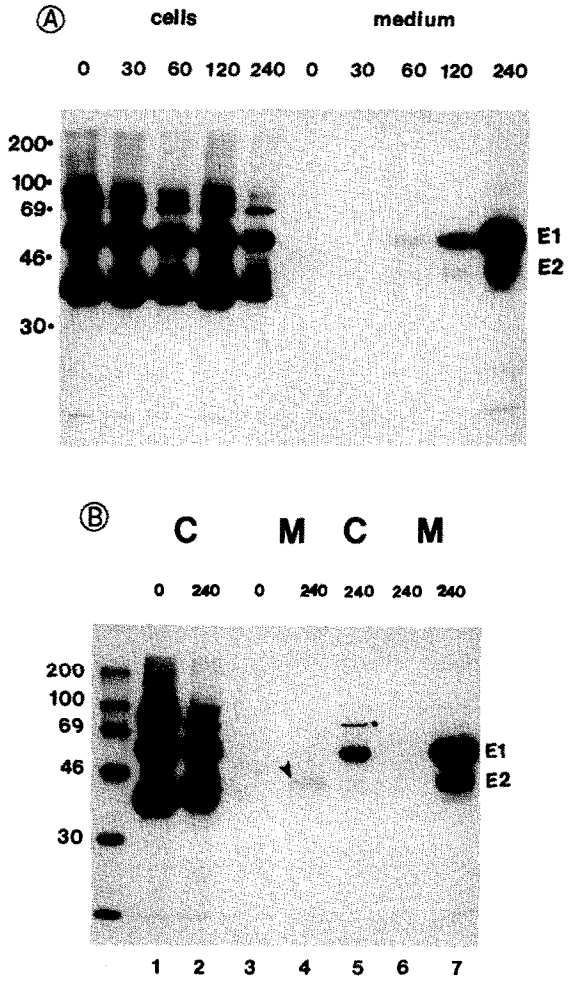

Fig. 4. E1ΔTM is secreted from COS cells when coexpressed with E2. Cells transfected with pCMV5-E2E1ΔM were pulsed for 30 min and chased for the indicated time periods before immunoprecipitates were prepared. (a) Time-dependent secretion of E1ΔTM. In this gel, 25% of the intracellular immunoprecipitates compared to the whole of the medium immunoprecipitates. Relative positions of E1 and E2 are shown and protein standards are indicated in kDa. (b) Most of E2 and E1 remain cell-associated after four hours of chase. C = cell lysate immunoprecipitates; M = immunoprecipitates from chase medium. Cells were transfected with pCMV5-E2E1 (lanes 1, 2, 3, 4), pCMV5-E1ΔTM (lanes 5, 6) and pCMV5-E2E1ΔTM (lane 7). Some E2 is present in the medium of pCMV5-E2E1 expressing cells (arrowhead lane 4). A dot indicates BiP which coprecipitates with intracellular forms of RV glycoproteins.

labelling experiment with one of the stable E1-secreting cell lines, CHO-E2E1ΔTM. The autoradiograph was scanned with a densitometer and it was found that after one, two and four hours of chase, 5%, 18% and 28% respectively, of the total E1 was found in the medium (Fig. 5). Judging by the heterogeneity of the E2 and E1

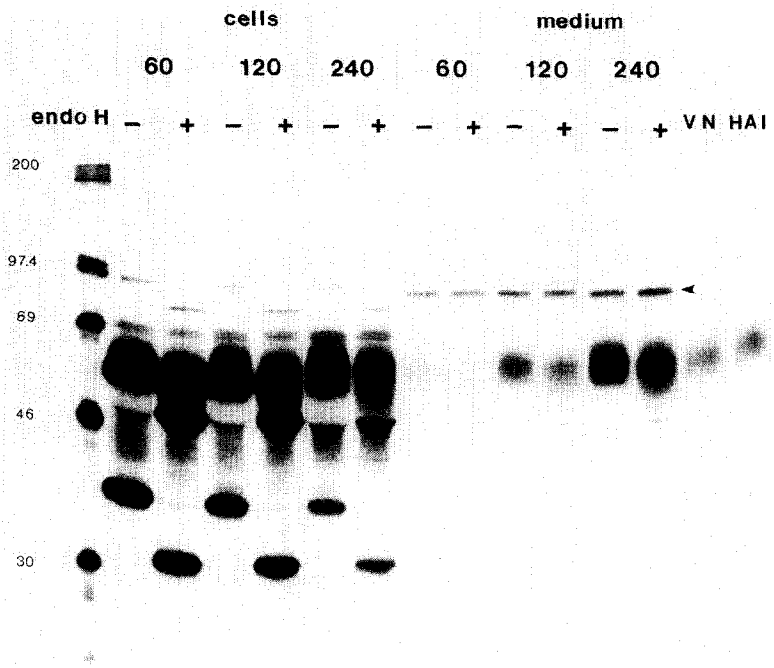

Fig. 5. Secretion of RV E1 from stably transfected CHO cells. CHO-E2E1ΔTM cells were pulse-labelled with 100 μCi [^{35}S]methionine for 30 min and chased for indicated times with media containing excess unlabelled methionine. RV proteins were recovered from cell lysates or media by radioimmunoprecipitation with human anti-RV serum or anti-E1 monoclonals (HAI and VN lanes). Immunoprecipitates were split into two parts and incubated at 37°C with or without 2 mU endo H glycosidase for at least 12 h. Equal amounts of immunoprecipitates from cell lysates and media samples were electrophoresed by SDS-PAGE. [^{14}C]-labelled molecular weight standards (kDa) were included for reference. Viral neutralizing (VN) antibody 21B9H, and hemagglutination inhibiting (HAI) antibody H4C52 were used to precipitate 4-h chase media from labelled CHO-E2E1ΔTM cells. The identify of the 85–90 kDa band precipitated with human anti-RV serum (arrow) is unknown.

bands (Fig. 5), RV glycoproteins expressed in CHO cells appeared to be more heavily glycosylated than those expressed in COS cells. Very little E2 was detected in the medium of transfected CHO cells. Golgi processed E2 from CHO cells migrated more heterogeneously on SDS-PAGE than E2 from COS cells. Consequently, it was more spread out and more difficult to detect by autoradiography. Therefore, there may be just as much E2 secreted from CHO cells, but because of the heterogeneous glycosylation, it was not easily visible in the medium. Although 28% of the E1 was secreted after four hours, approximately 60% of the cell-associated E1 was resistant to endo H glycosidase. Whether this fraction of E1 is retained in the Golgi apparatus or on the cell surface in complex with membrane-anchored E2 has not been determined. Additionally, another protein of 85–90 kDa was found to coprecipitate with E1 from the culture medium, but was not E1-specific since it was not recognized by two monoclonal antibodies to E1 (arrow,

Fig. 5). The identity of this protein remains to be determined. Lastly, the ER protein, BiP (Bole et al., 1986; Hobman et al., 1990) was not observed to precipitate with intracellular RV antigens expressed in CHO cells.

4. Discussion

At present, serological techniques with whole RV as target antigens for the detection of antibodies to RV are most commonly used for laboratory diagnosis of acute and congenital rubella infections and for determination of rubella immunity. These assays lack defined specificity against individual structural proteins and it is not known which RV antigens are detected in the assays. Katow and Sugiura (1985) observed that antibodies to E1 protein were predominant following most RV infections. In contrast, antibodies to E2 protein were relatively more abundant in congenital rubella syndrome than in other forms of RV infection. Thus it may be necessary to use antigenic forms of individual RV structural proteins in serodiagnostic assays. In this regard, stable cell lines expressing high levels of secreted E2 and E1 may prove to be convenient sources of E2 and E1 for serological diagnosis of RV immune status as well as RV vaccine development.

In this study, we have converted the normally membrane-bound RV glycoproteins E2 and E1 into secreted forms using a transient expression system. Although E2 is normally present as a member of the heterodimeric spike complex, it did not require E1 for transport to the cell surface in its soluble form and behaves like previously described secretable forms of viral glycoproteins (Gething and Sambrook, 1982; Rose and Bergmann, 1982). In contrast, E1ΔTM protein was retained intracellularly when expressed in the absence of E2 (Fig. 2). Secreted E1ΔTM protein from CHO-E2E1ΔTM retained the capacity to bind to anti-E1 monoclonal antibodies with VN and HAI properties (Fig. 5), demonstrating that the protein retains epitopes expected to be important properties of a subunit vaccine against RV. VN and HAI epitopes on E1 have been mapped between amino acids 245–285 by Terry et al. (1988) and 202–285 by Wolinsky et al. (1991). It is probable that this region of the protein may have acquired its native tertiary structure by the time the C-terminus of E1ΔTM protein is released into the lumen of the ER. Whether or not E1ΔTM protein can elicit the production of VN and HAI antibodies remains to be determined.

5. Acknowledgements

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