Envelope Proteins of Vesicular Stomatitis Virus: Effect of Temperature-Sensitive Mutations in Complementation Groups III and V

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All five major viral proteins were synthesized in chicken embryo cells infected with vesicular stomatitis virus temperature-sensitive (*ts*) mutants of complementation groups III and V and maintained at the nonpermissive temperature. The distribution of these proteins among cytoplasmic cellular fractions separated on discontinuous sucrose gradients was identical for wild-type and *ts*III-infected cells. Strikingly different patterns were observed for the G protein in gradients from cells infected by *ts*V mutants; very little, if any, G protein was found in the lightest fraction. Pulse and chase experiments with wild-type, virus-infected cells showed that protein G moves from the heaviest to the lightest fraction before being incorporated into the virion. After shift down to the permissive temperature (30 C), G protein synthesized at 39.6 C in *ts*V-infected cells became associated with the lightest cellular fraction and later with the released virions. In contrast, M protein, synthesized at 39.6 C in *ts*III-infected cells, was not incorporated into the virions after shift down. These data strongly suggest, first, that M protein is encoded by the vesicular stomatitis gene III, and second, that incorporation of G protein in the lightest cellular fraction is a necessary step of vesicular stomatitis maturation. This step is impaired by *ts*V mutations.

The vesicular stomatitis virion contains at least five proteins designated according to Wagner et al. (23): L, G, N, NS, and M (12, 24). These proteins constitute the major proteins found in cytoplasmic extracts of infected cells (13), though additional viral minor components may be present (1, 19, 20, 25).

These extracts can be fractionated by high-speed centrifugation into soluble and insoluble components. NS and half of the N are recovered in the soluble fraction, and the majority of M and G and half of the N are recovered in the insoluble one (25). Association of viral proteins with cellular insoluble components has been investigated by several methods (4-6, 8). Wagner et al. (22) observed an unequal distribution of the viral proteins among four cellular fractions which can be separated on discontinuous sucrose gradients. The lightest one contains mainly protein G, whereas protein N is the major viral component of the third fraction.

In this paper we show that temperature-sensitive (*ts*) mutations classified in complementation group V (10, 11) specifically affect the association of the G protein with the lightest cellular fraction at the nonpermissive temperature.

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MATERIALS AND METHODS

Virus, cells, and media. Wild-type *ts*⁺ strain and the following spontaneous *ts* mutants of vesicular stomatitis virus, serotype Indiana, described by Lafay (15-17) and Flamand (10), have been used: *ts*V 44, *ts*V 45, *ts*V 57, *ts*V110, and *ts*III 89.

Chicken embryo cells were grown in Eagle minimal essential medium buffered with Tris and supplemented with 6% calf serum and 8% tryptose phosphate.

Chemicals. Calf serum, Eagle minimal essential medium, and special Eagle minimal essential medium without leucine were purchased from Laboratories Eurobio. Ultrapure Tris was obtained from Schwartz/Mann. [³H]leucine (30 Ci/mM) and [¹⁴C]amino acid mixture (50 mCi/mg) were obtained from Commissariat à l'Energie Atomique (CEA-France). Actinomycin D was a generous gift of Merck, Sharp and Dohme (French branch).

Labeling of chicken embryo cells or virus. Monolayers of chicken embryo cells in 9-cm petri dishes were infected at a multiplicity of 40 to 150 PFU/cell. Virus was adsorbed for 45 min at 17 C, and the cells were incubated at 30 C in Eagle medium. Actinomycin D (1 µg/ml) was used when the multiplicity of infection was less than 100 PFU/cell. Three hours

after infection, the medium was removed, and pre-warmed special minimal essential medium without unlabeled leucine and containing $10 \mu\text{Ci}$ of [^3H]leucine per ml was added. Plates were immediately set either at 39.6 or at 30°C for the labeling period.

Cell fractionation. Whereas four to eight petri dishes of cells are sufficient to give enough radioactive material, in order to obtain visible light-scattering bands, carrier unlabeled cells were added. For this reason, 15 monolayers were used for each gradient. The procedure of fractionation was basically that of Caligiuri and Tamm (3), already applied to vesicular stomatitis virus by Wagner et al. (22). After washing with cold Tris-buffered saline, cells were scraped, centrifuged, washed again, and recentrifuged at $1,500 \times g$ for 5 min. The pellet was suspended in 5 ml of reticulocyte standard buffer, consisting of 0.01 M Tris, 0.01 M NaCl, and 0.0015 M MgCl_2 (pH 7.4). After allowing the cells to swell for 20 min, the cells were disrupted with 20 strokes of a Dounce homogenizer. Nuclei and large cell debris were removed by centrifugation at $800 \times g$ for 5 min. The cytoplasmic suspension, containing membrane fragments, was fractionated by centrifugation in a discontinuous sucrose gradient of the following composition: 3 ml of 60% sucrose in reticulocyte standard buffer, 7 ml of 45%, 7 ml of 40%, 10 ml of the cytoplasmic suspension in 30% sucrose, 7 ml of 25%, and 3 ml of reticulocyte standard buffer. Gradients were centrifuged at $86,000 \times g$ for 18 h in a Spinco SW27 rotor. Four light-scattering bands appeared at the interfaces of the successive layers, except between the 0 to 25% layers. The bands were collected from the side of the tube with a syringe. The pellet fraction was also recovered. Each fraction diluted with reticulocyte standard buffer was repelleted by centrifugation at $80,000 \times g$ for 90 min in a Spinco type 30 rotor. Soluble proteins did not migrate in the gradient and were recovered by trichloroacetic acid precipitation from the 30% sucrose layer and from the supernatants of the repelleted fractions 1 and 2.

Extraction and analysis of proteins. Cytoplasmic extracts and virions were treated by the Maizel method (18). After adding 0.1 vol of glacial acetic acid, the suspension was made 0.5 M with respect to urea and 1% with respect to sodium dodecyl sulfate. After incubation for 1 h at 37°C , samples were dialyzed overnight at room temperature against 1,000 vol of 0.01 M phosphate buffer (pH 7.2) containing 0.1% sodium dodecyl sulfate, 0.1% 2-mercaptoethanol, and 0.5 M urea. Cytoplasmic solubilized proteins were mixed before electrophoresis with 10 μl of marker ^{14}C proteins extracted from purified vesicular stomatitis virions (21). Electrophoresis was performed as previously described (16), except that a higher gel concentration (7.5% acrylamide) was used. Frozen gels, sliced into 1-mm disks, were depolymerized in 0.2 ml of 60 vol of H_2O_2 at 60°C for 4 h prior to the addition of Bray scintillation fluid (2). Vials were counted in an Intertechnique SL40 assisted by a double-labeling computer program.

RESULTS

Thermolability. Among all the *ts* mutants of the Orsay collection, the virions of *tsV* 45 and

tsV 110 are the more thermolabile in vitro. Both belong to complementation group V. Thermal inactivation of *tsV* 45 has already been described by Deutsch and Berkloff (9). The two other mutants classified in group V, *tsV* 44 and *tsV* 57, show the same thermolability as the reference wild-type strain (Fig. 1).

Viral proteins in whole cytoplasmic suspension of infected chicken embryo cells. The distribution in a sodium dodecyl sulfate-polyacrylamide gel of the viral proteins synthesized at 39.6°C in cells infected either with the wild-type or the *tsV* 45 strains are shown in Fig. 2. As already noted by Printz and Wagner (21) and by Wunner and Pringle (26), no significant differences appear between the two profiles. Under these conditions, proteins N and NS migrate very closely and are not generally resolved as two well-separated peaks. In Fig. 2 the NS peak is separated by only one point from *tsV* 45 and is not distinct from the N peak for the wild type. Such a difference is not significant. As noted by Kang and Prevec (13), the virion marker G protein migrates slower than the intracellular G. Compared to results obtained at 37°C with L cells (13, 25) or BHK-21 cells (26), we regularly find a smaller relative amount of M protein. Cells infected at 39.6°C with *tsV* 57, *tsV* 110, and *tsIII* 89 give patterns identical to those of Fig. 2.

Distribution of viral proteins in cytoplasmic fractions from infected chicken embryo

FIG. 1. Thermolability of *tsV* mutant strains. Samples (1 ml) of fresh virus stocks were immersed in a water bath at $40^\circ\text{C} \pm 0.1$ and periodically assayed for survival of plaque-forming ability on chicken embryo cells at 30°C . Symbols: ■, *ts*⁺; □, *tsV*44; △, *tsV*45; ○, *tsV*57; ●, *tsV*110.

FIG. 2. Proteins synthesized at nonpermissive temperature in chicken embryo cells infected either with the ts^+ wild-type or the tsV 45 strains. Chicken embryo cells are infected at a multiplicity of infection of 80 PFU for the wild type (●-●) and of 120 PFU for tsV 45 (○-○). After an adsorption period of 45 min at 17 C, cells were incubated at 30 C for 3 h in medium without (tsV 45) or with (ts^+) 1 μ g of actinomycin D per ml. [3 H]leucine was then added and the infected cells were further incubated at 39.6 C for 3 h. Cells were then washed, scraped, pelleted, and disrupted in reticulocyte standard buffer. Cytoplasmic extracts were prepared as described in Materials and Methods. Electrophoresis was performed for 5 h on 7.5% sodium dodecyl sulfate polyacrylamide gels. The arrows show the peak position of the 14 C-coelectrophoresed vesicular stomatitis structural proteins.

cells. The distribution of viral proteins L, G, N, and M, synthesized at 39.6 C in five cytoplasmic fractions of infected cells separated on a discontinuous sucrose gradient, is shown in Fig. 3. In the wild-type infection, G protein is the major viral component of band 1, which contains 20% of the radioactive-insoluble G protein. N protein is regularly present in small amounts, whereas M protein is not always found. G is also predominant in bands 2 and 3, where the three other proteins are always found. N is predominant in band 4 and in the pellet, which, together, contain two-thirds of the radioactivity associated with sedimentable material. We do not find M in the pellet. The distribution of each protein in the different fractions and of the different proteins in each fraction is given in Table 1, which includes results for soluble material.

Extracts of cells infected either with ts^+ or with tsV 45, tsV 57, or tsV 110 and incubated at 30 C give similar distribution. Strikingly different patterns are obtained for G protein synthesized at 39.6 C in cells infected by the tsV mutant strains, tsV 110 (Fig. 3), tsV 45 (Fig. 6), and tsV 57 (not shown). Very little G protein is

found in band 1, and the proportion of G protein in band 2 is often reduced. Results obtained from cells infected at 39.6 C by the $tsIII$ 89 strain are similar to those observed with the ts^+ wild type (Table 2). Thus, it would appear that the abnormal distribution of G protein is a characteristic of group V mutants.

Distribution of viral pulse-labeled proteins in cytoplasmic fractions. In order to provide information on the virus biogenesis, we have studied the evolution of ts^+ wild-type, viral pulse-labeled proteins in the cytoplasmic fractions of a discontinuous sucrose gradient.

At the end of a 10-min labeling period with [3 H]leucine (10 μ Ci/ml), the majority of the radioactivity found in the cells is not trichloroacetic acid-precipitable. This soluble radioactivity decreases very rapidly during the first 10 min of incubation in Eagle medium containing 100 μ g of non-radioactive leucine per ml. It is clear from Fig. 4 that the bulk of the soluble radioactivity in the cells at the end of a pulse is not ultimately incorporated in the trichloroacetic acid-insoluble material, attesting to the chase efficiency.

Fractionation in sucrose gradients of cytoplasmic extracts was performed immediately after pulse-labeling for 20 min (t_0) and after 20 (t_{20}) and 45 min (t_{45}) of incubation at 37 C in chase medium. The virions present in the culture supernatant and the soluble proteins recovered from the gradient were also analyzed. The results are summarized in Fig. 5. The virion-associated radioactivity increases from 5% of the total label at t_0 to 15% at t_{20} and 33% at t_{45} , but the kinetics of incorporation of radioactivity into the virions is very different from one protein to another. Half of the labeled M protein is already incorporated into virions at t_0 , 80% at t_{20} , and almost 100% at t_{45} . No detectable labeled G protein is found in the virions at t_0 and t_{20} and only 12% is found at t_{45} . The proportion of labeled G protein increases in band 1 from 2% at t_0 to 4% at t_{20} and 10% at t_{45} . No significant variation in the proportion of G is observed in the pellet and in fractions 2, 3, and 4. Soluble proteins, which are essentially N and NS, decrease from 20% at t_0 to 5 to 6% at t_{20} and t_{45} . Concomitantly, the proportion of N label increases first in the pellet and later in the virions.

Distribution among cytoplasmic fractions of the tsV viral proteins synthesized at 39.6 C after shift down to 30 C. Cells infected with tsV mutants were incubated at 39.6 C from 3 to 6 h after infection in medium containing 10 μ Ci of [3 H]leucine per ml. The distribution of viral

FIG. 3. Distribution of viral proteins in cytoplasmic fractions of infected cells separated on discontinuous sucrose gradient. After a 45-min period of adsorption at 17 C, infected cells were incubated for 3 h at 30 C in the presence of actinomycin D (1 μ g/ml). [3 H]leucine (10 μ Ci/ml) was then added, and cells were further incubated for 3 h at 39.6 C. The cells were then disrupted and centrifuged at $800 \times g$ to remove nuclei. The supernatant was centrifuged for 18 h at $86,000 \times g$ in a discontinuous 0 to 60% sucrose gradient. Light-scattering bands, located at the interfaces of the successive sucrose layers, were collected and pelleted by centrifugation at $80,000 \times g$ for 90 min. Fraction 1 is located at the 25 to 30% interface; fraction 2 at the 30 to 40% interface; fraction 3 at the 40 to 45% interface, and fraction 4 at the 45 to 60% interface. Left panel: wild type (multiplicity of infection [MOI] = 60); middle panel: tsIII 89 (MOI = 30); right panel: tsV 110 (MOI = 40).

proteins labeled at 39.6 C was analyzed after 0, 20, and 40 min of incubation at 30 C in non-radioactive chase medium (Fig. 6). During the labeling period, there was no release of PFU to the supernatant. After the temperature shift down, virion release began within 30 min, and extracellular particles accumulated rapidly (Fig. 7).

The maturation of G protein synthesized at nonpermissive temperature during the chase period at 30 C is summarized in Fig. 6. As already seen, very little radioactive G is present in band 1 at the end of the 3-h labeling period at 39.6 C. The amount of labeled G in band 1

increases progressively during postincubation at 30 C and appears in virions. Thus, the G protein synthesized at the nonpermissive temperature in cells infected by tsV mutants is able, after a temperature shift down, to be incorporated first into the cellular fraction 1 and later into virions.

Appearance of proteins synthesized at 39.6 C in tsIII and tsV virions after shift down at 30 C. In order to see if radioactive G protein synthesized at the nonpermissive temperature is normally incorporated into virions after a temperature shift down, we have compared the 3 H- 14 C ratio for each structural protein of groups III and V ts mutants. [3 H]leucine

TABLE 1. *Distribution of the radioactivity associated with G, N (+NS for the soluble fraction), and M proteins in the fractions isolated from a discontinuous sucrose gradient^a*

Band	Protein ^b						Label in each fraction (%)
	G		N		M		
	Distribution in six fractions (%)	Protein in each fractions (%)	Distribution in six fractions (%)	Protein in each fraction (%)	Distribution in six fractions (%)	Protein in each fraction (%)	
1	19	95	0.2	2	5	3	6
2	27	58	6	33	36	9	13
3	7	61	1	28	10	11	3
4	22	31	17	60	50	8	20
Pellet	8	18	14	82	ND ^c	ND	12
Soluble	16	9	61	91	ND	ND	47

^a Chicken embryo cells were infected with wild type. The experimental protocol is given in the legend to Fig. 3.

^b Percent of label in each protein: G, 27; N, 70; M, 3.

^c ND, Not detectable.

FIG. 4. Efficiency of the chase medium. Cells infected with *ts*⁺ wild type (multiplicity of infection = 100) were incubated at 37 C. [³H]leucine was added 4 h after infection. Ten minutes later, cells were washed several times with cold Tris buffer and covered with Eagle medium supplemented with 100 μg of unlabeled

TABLE 2. *Distribution of G protein synthesized at nonpermissive temperature in different cytoplasmic fractions^a*

Band	Complementation group					
	<i>ts</i> +		<i>ts</i> III 89	<i>ts</i> V 110	<i>ts</i> V 45	
	1	2			1	2
1	18	23	18	2.5	2	3
2	38	32	31	17	22	40
3	11	9	20	12	9	14
4	17	26	17	31	33	30
Pellet	17	10	17	37	33	13
Fraction ^b	35	35	25	16	25	15

^a Expressed as the percentage of G in each fraction. Experimental procedures were as described in legend to Fig. 3.

^b Fraction of the total radioactivity incorporated in the viral proteins associated with G.

leucine per ml. The zero time sample was taken at this point, and subsequent samples were taken at 10-min intervals. Each sample was washed carefully, and cells were treated with 5% trichloroacetic acid. The amounts of radioactivity in the trichloroacetic acid-soluble (O-O) and precipitable (●-●) fractions were determined and plotted.

FIG. 5. Distribution of the radioactivity associated with L, G, N (+ NS for the soluble fraction), and M in the fractions (frctn) of a sucrose gradient and in the virions. Each figure represents the percentage of the total radioactivity recovered in each gradient. Chicken embryo cells were infected with wild type and pulse-labeled for 20 min. at 4 h postinfection. Incubation temperature was 37 C. (A) Chase 0 min; (B) chase 20 min; (C) chase 45 min.

was present during incubation at 39.6 C (3 to 6 h postinfection), washed out just before shift down at 30 C, and replaced by a [^{14}C]amino acid mixture. Extracellular virions were collected after 1.5 h of additional incubation. The *tsIII* mutant was a better control than *ts+* wild type, because maturation was blocked for both groups at 39.6 C and began within the same time after shift down to 30 C. The relative incorporation into virions of proteins G, N, and

M synthesized at the nonpermissive (^3H labeled) and at the permissive (^{14}C labeled) temperature is shown in Fig. 8. The ^3H - ^{14}C ratios in proteins G and N are identical for the two mutants. Thus, the G protein synthesized at 39.6 C in cells infected by *tsV* mutants is therefore incorporated into virions in normal amounts after shift down at 30 C. The M protein is synthesized at 39.6 C by *tsIII* 89-infected cells and easily detected in cytoplasmic

FIG. 6. Evolution after a shift down at 30 C of the distribution among cytoplasmic fractions of the *tsV* 45 viral proteins synthesized at 39.6 C. Cells were infected with *tsV* 45 (multiplicity of infection = 120) in the same way as described in Fig. 2. After the 3-h labeling period at 39.6 C, the cells were further incubated in unlabeled chase medium at 30 C for 20 or 40 min before collection. Labeled virus, released into the supernatant only after 40 min of incubation at 30 C, were also collected. Fractionation was carried out on sucrose discontinuous gradients as described in Fig. 3.

extracts (Fig. 3), but it is not recovered into virions produced after the temperature shift down, as already reported for *tsIII* 23 (16). The high value of the ³H-¹⁴C ratio of M protein in the *tsV* 45 virions is also regularly observed in wild-type infection for such labeling periods. This may be due to the fact that protein M is incorporated into virions very quickly after

synthesis, as already shown in Fig. 5 and by Kang and Prevec (13).

DISCUSSION

The viral proteins L, G, N, and M are found in the same proportion in the whole cytoplasmic extracts from cells infected at permissive or nonpermissive temperature either by the wild-

FIG. 7. Growth curves at 30, 39.6, and 30-39.6-30 C shift of mutant *tsV 45*. After a 45-min period of adsorption at 17 C, cells infected with *tsV 45* (multiplicity of infection = 5) were washed with Tris buffer. Fresh medium was added and petri dishes were held at 30 or 39.6 C. One hour later the medium was discarded and replaced by prewarmed medium; 3 h after infection one plate at 30 C was transferred to 39.6 C for 3 h and finally set back at 30 C. At the indicated times samples were taken for titration. Symbols: ○, 30 C throughout; ▲, 39.6 C throughout; ●, 30-39.6-30 C; —, 30 C incubation; - - -, 39.6 C incubation.

type or by *ts* mutants of groups III and V. This is not unexpected, since thermosensitivity is usually the consequence of mis-sense mutations which have no effect on the gene translation and whose expression at the polypeptide level is the substitution of one amino acid by another. Such a substitution, which could affect the protein configuration and function at nonpermissive temperature, is not expected to modify the migration properties of the polypeptide in a sodium dodecyl sulfate-polyacrylamide gel.

We interpret the results of our pulse-chase experiment with wild-type-infected cells as meaning that the vesicular stomatitis G protein migrates from the heaviest fractions to the band 1 before being incorporated into the virions. Migration for influenza glycoproteins through the cellular fractions seems quite similar (7, 14). As already noted by Kang and Prevec (13), we observe that newly synthesized M protein is very quickly incorporated into the virions. In accord with Wagner et al. (25), we found that N protein migrates from the soluble to the heaviest fractions, probably as a result of nucleocapsid formation.

This work demonstrates that association of G

protein with the lightest insoluble fraction which can be resolved by discontinuous sucrose gradients is blocked at the nonpermissive temperature by all four mutations classified in complementation group V by Flamand (10). It is important to notice that identical results are obtained either with group V mutants which are thermolabile in vitro or with those which are not. The simplest interpretation of these observations is to assume that incorporation of G protein in cellular fraction 1 is a necessary step of vesicular stomatitis virus maturation.

After transfer to the permissive temperature, the G protein synthesized at the nonpermissive temperature becomes associated with the light cellular fraction and released virions. This result does not allow a choice between two hypotheses: either gene V is the structural gene for the G protein or gene V encodes a minor protein necessary for G incorporation in cellular fraction 1. If gene V is the structural gene of protein

FIG. 8. Polyacrylamide gel electrophoresis of *tsIII 89* and *tsV 45* virions released after a shift down at 30 C. Cells infected with *tsV 45* (multiplicity of infection [MOI] = 20) or *tsIII 89* (MOI = 15) were incubated at 39.6 C from 3 to 6 h after infection in Eagle medium containing 2.5 μ Ci of [3 H]leucine per ml. Cells were washed, and minimal essential medium diluted 10-fold in Tris-saline containing 2.5 μ Ci of [14 C]amino acid mixture per ml and 100 μ g of unlabeled leucine per ml was added. Virions were collected after 1.5 h of incubation at 30 C and pelleted at $80,000 \times g$ for 60 min, and proteins were extracted as described in Materials and Methods. Top panel: *tsV 45*; lower panel *tsIII 89*. Symbols: ●, 3 H label; ○, 14 C label.

G, we must conclude that alterations produced by the four *tsV* mutations in the G protein synthesized at 39.6 C are reversible upon shift down or, alternatively, that the G protein synthesized after shift down allows G protein synthesized at 39.6 C to be incorporated into fraction 1.

Protein M, synthesized at 39.6 C by *tsIII* 89, is readily detected in cytoplasmic extracts, but, in contrast to group V mutants, it is not incorporated into virions after transfer to the permissive temperature. These results strongly confirm our previous hypothesis that gene III is the structural gene of the M protein (16, 17).

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