Two-Dimensional Electrophoretic Analysis of Encephalomyocarditis Viral Proteins

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All four capsid proteins of encephalomyocarditis virus and the precursor to two of these were resolved from purified virions with two-dimensional gel electrophoresis. In addition, all of the known stable virus-specific proteins found in infected cells, but not the primary and intermediate precursor proteins, could be resolved with these techniques.

Two-dimensional gel electrophoresis is a relatively new technique that has been used extensively in the last several years (12, 13). Proteins are separated in the first dimension by charge in a polyacrylamide gel containing a pH gradient and protein solubilizers and in the second dimension by molecular weight in a polyacrylamide gel that contains sodium dodecyl sulfate (SDS). Several investigators have studied the properties of picornaviral capsid proteins from Mengo virus (2), poliovirus (5-7, 22, 23), and foot-and-mouth disease virus (8) with pH gradient separation. We present here our work with encephalomyocarditis (EMC) viral capsid proteins and, in addition, work we have done with noncapsid proteins of this virus with two-dimensional gel electrophoresis.

Isoelectric focusing (IEF) and non-equilibrium pH gradient electrophoresis (NEPHGE) were done basically as described by O'Farrell et al. (12, 13). Both IEF and NEPHGE gels were equilibrated in 10% (vol/vol) glycerol-2% (wt/vol) SDS-0.075 M Tris-sulfate (pH 9.0)-1% (vol/vol) saturated bromophenol blue for 2 h before being secured on top of a second-dimension slab gel with 1% (wt/vol) agarose. Separation in the second dimension was with the discontinuous SDS-polyacrylamide slab gel system of Tegtmeier et al. (20).

Three of the four EMC viral capsid proteins, α , β , and δ , and the precursor to β and δ , ϵ , were resolved from purified virus samples when IEF was used for the first dimension of two-dimensional gel electrophoresis (Fig. 1A). Both α and β had one major isoelectric species and a minor component that was more acidic. The isoelectric points of the major species of α and β were estimated as 6.9 and 6.6, respectively (Fig. 1A). Another film exposed to the gel shown in Fig. 1A, where the optical density of spots α and β did not exceed 1.5, was scanned with a Gilford model 2520 gel scanner. We estimated the quantity of material in each minor band correspond-

ing to α and β to be less than 15% of that in the respective major bands. Capsid protein δ was detected only after a long exposure time (2 weeks) (Fig. 1A) as a faint spot with an estimated isoelectric point of 6.4. An appreciable amount of this low-molecular weight protein was probably lost from the first-dimension gel during the equilibration step before separation in the second dimension (5, 7). A trace of the uncleaved capsid precursor protein ϵ was present in this virus preparation and could be seen in the two-dimensional profile as a faint spot with an estimated isoelectric point of 6.1, a value more acidic than those of its cleavage products β (6.6) and δ (6.4). Perhaps a small acidic peptide is cleaved from ϵ at the same time as the cleavage that forms β and δ . Alternatively, ϵ , β or δ might not be completely denatured in the sample solution of urea, Nonidet P-40, SDS, and 2-mercaptoethanol, and some charges might then be shielded during IEF.

Capsid protein γ was not resolved by IEF and SDS-polyacrylamide gel electrophoresis. Purified virus samples were subjected to NEPHGE to see if γ was a basic protein. Capsid proteins α , β , and ϵ were resolved in a more compressed pattern when NEPHGE rather than IEF was used in the first dimension. Protein γ was resolved at the basic end of the gel and likely had an isoelectric point greater than 7.5.

There appears to be no similarity in the acid-base properties of α , γ , or δ and the analogous capsid proteins from poliovirus (5-7) and foot-and-mouth disease virus (8). Only capsid protein β (analogous to VP2) has a similar isoelectric point in all three picornavirus species.

Functional EMC viral proteins arise through the cleavage of a series of precursor polypeptides (18). To produce radiolabeled precursors and many of the product proteins, HeLa cells infected with EMC virus were given a 40-min pulse of [3 H]leucine. As host cell synthesis is blocked soon after infection with EMC virus, most of the

FIG. 1. Fluorograms of two-dimensional gels containing EMC viral capsid proteins. Production of EMC virus at 37°C in suspension cultures of HeLa cells was done essentially as previously described (17), except that virions were labeled in the presence of 15 μ Ci of L-[4,5- 3 H]leucine per ml from 3 to 8 h after the addition of virus to cells. Purification of virus by centrifugation through a cushion of 30% (wt/vol) sucrose, density gradient centrifugation in CsCl of the resuspended viral pellet, and gel filtration through a Bio-Gel A-5m (100 to 200 mesh) column was done essentially as described by Medappa et al. (11). Samples were prepared for IEF or NEPHGE by adding 0.054 g of crystalline urea and 2 μ l (0.8 U) of bovine pancreatic RNase I (EC 3.1.4.22) at 5 mg/ml to 59 μ l of purified virus. After a 1-h incubation at 25°C, 2 μ l of Nonidet P-40, 2.5 μ l of 10% (wt/vol) SDS, and 5 μ l of 2-mercaptoethanol were added. In all figures, reference samples prepared as previously described (17) were placed on the side of the SDS-polyacrylamide gel in a small notch 2 to 3 mm lower than the surface where the first-dimension gel was placed. As a result, the reference proteins appear to have a greater mobility than the respective spots on the second-dimension gels. (A) IEF was carried out for 20 h at 400 V. (B) NEPHGE was carried out for 4 h at 400 V. Electrophoresis in the second dimension after IEF or NEPHGE was carried out for 18.5 and 18 h, respectively, at 7.5 mA. Gels in all figures were prepared for fluorography (1) and exposed to Kodak XR-5 film as previously described (10).

radiolabeled proteins are virus-specific (3). Two-dimensional electrophoretic analysis of these proteins with IEF in the first dimension resolved noncapsid proteins E, F, and H, capsid protein

α , and the capsid precursor protein ϵ (Fig. 2A). Noncapsid protein H separated into two distinct spots, designated H and H1, of comparable intensities, with slightly different molecular weights and isoelectric points. The two proteins could be separate gene products or they could be closely related. Pallansch et al. (14) have shown that amino acid sequences of the VPg protein of EMC virus are contained within protein H. Recent evidence indicates the VPg is present in two different forms in foot-and-mouth disease virus (9) and EMC virus (21). The separation of H into two distinct spots by two-dimensional gel electrophoresis may be a reflec-

FIG. 2. Fluorograms of two-dimensional gels containing virus-specific polypeptides from HeLa cells infected with EMC virus. IEF was used in the first dimension. The infected cells were prepared essentially as described previously (17) but all incubations here were at 37°C. At 3.5 h after cells and virus were combined, L-[4,5- 3 H]leucine was added to give 25 μ Ci/ml, and the culture was divided into several portions. The pulse portion of the culture (A) was prepared for IEF 40 min after addition of [3 H]leucine. The pulse-chase portion of the culture (B) was allowed to incubate for 20 min after the addition of [3 H]leucine, followed by a 60-min chase initiated by the addition of a 1,000-fold molar quantity of unlabeled leucine. Samples were prepared for IEF by suspending 6×10^6 cells in 100 μ l of O'Farrell lysis buffer (12) modified by adding SDS to 0.5% (wt/vol). Reference samples were also prepared from portions of the pulse and pulse-chase samples. For both gels, IEF was carried out for 20 h at 400 V, and electrophoresis in the second dimension was carried out for 16.5 h at 7.5 mA.

tion of the two forms of VPg. When we used a 20-min pulse of [^3H]leucine followed by a 60-min chase of unlabeled leucine to radiolabel all of the final cleavage products, two additional proteins, capsid protein β and noncapsid protein I, were also resolved (Fig. 2B).

Many of the noncapsid viral proteins had both a major and a minor component as described earlier for the capsid proteins shown in Fig. 1A. Similar trace components are found for poliovirus (5-7) and foot-and-mouth disease virus (8) capsid proteins.

The isoelectric points of the noncapsid proteins were determined from gels shown in Fig. 2A and B and a third gel, and the average values and ranges (shown within parentheses) were as follows: E, 6.6 (6.2 to 6.9); F, 7.2 (6.7 to 7.6); H, 5.7 (5.6 to 5.8); H1, 5.5 (5.4 to 5.7); and I, 5.6 (5.4 to 5.7). Average isoelectric points were also determined for capsid proteins α and β and the capsid precursor ϵ from the gels shown in Fig. 1A and 2A and B and another gel containing proteins from infected cells. The values and their ranges (shown within parentheses) were as follows: α , 6.8 (6.2 to 7.0); β , 6.3 (6.0 to 6.6); and ϵ , 5.9 (5.7 to 6.1). Capsid protein δ was not detected in infected cell extracts.

A preparation of infected cells labeled with [^3H]leucine for 40 min was also subjected to NEPHGE to resolve basic viral proteins that might be outside the pH range used for IEF. This sample was also treated with S1 nuclease to degrade cellular nucleic acids that might interfere with the electrophoretic migration of the proteins. Three basic viral proteins, γ , G, and the recently described viral protease p22 (4, 15, 19), not resolved by the IEF procedure, were resolved by NEPHGE and were detected on the second dimension (Fig. 3). The isoelectric point of γ was probably greater than 7.5, as shown in Fig. 1B, and those of p22 and G were probably greater than 8. No proteins other than those already discussed were resolved when a pulse-chase preparation was subjected to identical treatment (data not shown).

We could not resolve the large-molecular-weight primary and intermediate precursors A, B, C, D, and D1, although the reference samples in Fig. 2A and 3 show they were present in the pulse preparations. There was, however, a group of faint spots located primarily between pH 5 and 6 and between the molecular weights of protein E (56,000) and protein A (100,000). If the cleavage of the large polypeptides occurs at a number of alternate cleavage sites, a number of protein species differing little in molecular weight but significantly in charge might be present. These would not be easily detected on two-dimensional gels. Alternatively, the large poly-

FIG. 3. Fluorogram of a two-dimensional gel containing virus-specific polypeptides synthesized in EMC viral-infected cells. NEPHGE was used in the first dimension. The infected cells were prepared and pulse-labeled for 40 min with L-[4,5- ^3H]leucine as described in the legend to Fig. 2A. Infected cells (6×10^6) were washed twice in phosphate-buffered saline and prepared for S1 nuclease digestion by the procedure of Peterson and McConkey (16). The sample was incubated for 5 min at 45°C after the addition of $7.2 \mu\text{g}$ (27 U) of S1 nuclease as recommended by Zechel and Weber (24). Ampholytes (pH 3 to 10), Nonidet P-40, and 2-mercaptoethanol were added to final concentrations of 2% (wt/vol), 2% (vol/vol), and 5% (vol/vol), respectively. NEPHGE was carried out for 4 h at 400 V. Electrophoresis in the second dimension was carried out for 16.5 h at 7.5 mA.

peptides may form insoluble aggregates under the conditions used for IEF and NEPHGE. We tried several modifications in our techniques to allow focusing of these large polypeptides but were unsuccessful in our efforts.

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