Pardaxin, a Hydrophobic Toxin of the Red Sea Flatfish, Disassembles the Intact Membrane of Vesicular Stomatitis Virus*

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Reaction of vesicular stomatitis virus with pardaxin, the hydrophobic toxin of the Red Sea flatfish, resulted in a profound morphological change of many virions and dissociation of their membrane and nucleocapsid into components readily separable by density gradient centrifugation. The basic matrix protein and acidic pardaxin segregated largely with the high density nucleocapsid. The dissociated virion membrane formed lipid-protein vesicles which retained glycoprotein spikes and a certain amount of N protein but no appreciable amounts of other nucleocapsid proteins and little if any RNA. Iodination of the tyrosine residue of the glycoprotein tail fragment provided supporting evidence that the COOH terminus of the glycoprotein extends beyond the inner layer of the membrane into the interior of the virion. These data indicate that pardaxin may serve as a probe for studying the organization of viral membranes, and, hopefully, other biological membranes.

Vesicular stomatitis virus is a membrane-enclosed, negative strand RNA virus that has been studied extensively as a model membrane system (1, 2). The nucleocapsid core comprises the RNA template closely associated with three proteins (N, L, and NS) which collectively comprise the RNA polymerase. The VS1 virion membrane contains an intrinsic glycoprotein, which is oriented externally (3), and a matrix protein, which apparently lines the inner surface of the virion bilayer (4) in close proximity to the nucleocapsid (5). A major problem encountered in studying the simple biological membranes of enveloped viruses is the inability thus far to remove the intact membrane of the virion, in a manner similar to that of erythrocyte membranes. We report here the potential use of the hydrophobic toxic protein, pardaxin, for dissociating the intact membrane of VS virus and possibly other biological membranes. Pardaxin, the ichthyolytic-hemolytic factor secreted from the skin of the Red Sea flatfish *Pardachirus marmoratus* (Soleidae), is an acidic protein of Mr ≈ 17,000 composed of 162 amino acids (6, 7). It is rich in hydrophobic amino acids, including leucine (18 residues), phenylalanine (13 residues), isoleucine (11 residues), and valine (5 residues) but is devoid of arginine, histidine, and tryptophan (6). Pardaxin exhibits strong interaction with lipid bilayers and makes vesicle membranes permeable as evidenced by leakage of entrapped 6-carboxyfluorescein. As previously described (8), plaque-purified VS virus of the Indiana serotype was used to infect baby hamster kidney-21 cells at a multiplicity of 0.1 plaque-forming units/cell. Homogeneous bullet-shaped virus harvested at 21 h postinfection was purified by differential, rate zonal, and equilibrium centrifugation in sucrose and tartrate gradients (5). [3H]Leucine- and [3H]uridine-labeled virions were prepared by the addition of 5 μCi/ml of [3H]leucine (55.9 Ci/mmol) or 5 μCi/ml of [3H]uridine (25.0 Ci/mmol) to the infection medium after initial adsorption of the virus to the cells. Phospholipids of the virion membrane were radiolabeled by VS virus infection of baby hamster kidney-21 cells prelabeled with 10 μCi/ml of [3H]palmitate and infecting them in the presence of 5 μCi/ml of [3H]palmitic acid (16.8 Ci/mmol) in ethanol.

Fig. 1 reveals that pardaxin profoundly alters the morphology of VS virions (50 μg) when incubated at 20 °C for 10 min with 20 μg of the purified toxin in 0.2 ml of phosphate-buffered saline, pH 7.4. Many of the typically bullet-shaped virions (Fig. 1A) assumed a spherical shape and a large proportion of the internal nucleocapsids were extruded (Fig. 1B). Dissociated virion membranes assumed the shape of large lipid vesicles surrounded by glycoprotein spikes.

We next attempted to isolate and characterize the VS virion membrane vesicles from which the nucleocapsid cores had been extruded by the action of pardaxin. To this end, three suspensions of VS virions (300 μg/ml each) were treated for 10 min with 120 μg of pardaxin and then made 50% with respect to sucrose; this suspension was overlayed with a 10-50% continuous gradient of sucrose and centrifuged in the SW 27.1 rotor at 62,400 × g for 16 h. To identify by density flotation the location of virion RNA, proteins, and lipids, separate samples of VS virus had been biologically labeled with [3H]uridine, [3H]leucine, or [3H]palmitate. As shown in Fig. 2, most of the [3H]palmitate floated to the top of the gradient at ρ = 1.06 g/ml but a substantial amount of [3H]palmitate banded at ρ = 1.15 g/ml, presumably representing undisrupted virions which banded at this same density (data not shown). In contrast, only ~20% of [3H]leucine-labeled viral protein floated to the top of the gradient, whereas the largest amount of protein banded at ρ = 1.2 g/ml, equivalent to that of delipidated nucleocapsids. The [3H]uridine label, representing viral RNA of pardaxin-extruded nucleocapsids, also banded at an equivalent density of 1.2 g/ml (data not shown). These experiments indicate that pardaxin dissociates VS virions into a lipoprotein fraction of low density and a ribonucleoprotein fraction of high density with a certain proportion of undisrupted virions of intermediate density.

Samples collected from the top three fractions of the sucrose gradient depicted in Fig. 2 were examined by negative stain electron microscopy. Fig. 1C reveals the presence of only low electron-dense paracrystalline particles with dense cores; many of these particles had closely associated with them a small fraction of extruded nucleocapsids.
Pardaxin Disassembles Intact VSV Membrane

FIG. 1. Electron microscopy by negative staining with phosphotungstic acid of (A) intact VS virions, (B) VS virions (50 μg) treated for 10 min with 0.2 ml of pardaxin (20 μg), and (C) lipid vesicles generated from VS virions (300 μg) treated with pardaxin (120 μg) and floated to the top of a 10-50% sucrose gradient by density centrifugation as shown in Fig. 2. Arrows point to vesicles (Ve) with spikes and to nucleocapsids (NC). × 140,000.

density vesicles, varying in size from large to very small resealed membrane fragments, all of which exhibit protruding spikes clearly analogous to those extending from the surface of intact virions (Fig. 1A).

The protein composition of pardaxin-treated VS virus lipid-rich fractions from the top of the gradient (ρ = 1.06 g/ml) and the RNA-rich fractions from the bottom of the gradient (ρ = 1.2 g/ml) was determined by electrophoresis on 12.5% polyacrylamide-sodium dodecyl sulfate gels. Gels stained with Coomassie blue were scanned by densitometry and the concentrations of each viral protein were determined by integration. Fig. 3 shows the electropherograms and Table I compares the relative concentrations of the five viral proteins in the low density and high density fractions with that of unfractionated virus. As noted, the high density nucleocapsid contains all five viral proteins but has a reduced concentration of G protein and was relatively enriched in N protein; as expected, this fraction was heavily contaminated with VS virions undisrupted by pardaxin, presumably accounting for the presence of G protein. By comparison, the low density lipid-rich fraction contained predominantly G protein. We are somewhat at a loss to explain why so much N protein was present in the low density vesicle fraction despite failure to detect appreciable amounts of nucleocapsid L and NS proteins. It is possible that pardaxin partitions into the hydrophobic regions of the nucleocapsid, thus dissociating some N protein to a monomeric form that can associate with membrane vesicles. We had previously shown that hydrophobic aryl azide probes covalently bind to N protein of intact VS virions (4). No intact nucleocapsids could be detected by electron microscopy of lipid-rich viral membrane vesicles dissociated with pardaxin. Most importantly, we could not detect any multilayer vesicles by electron microscopy which indicates that pardaxin does not release from the virion membrane appreciable amounts of free lipid that would form multilayer vesicles (bangasomes) under these conditions.

Of considerable interest is the observation that the M protein segregated with the nucleocapsids after pardaxin dissolution of virions and none could be detected in the low density virion vesicles (Table I). Moreover, most of the M, = 17,000 pardaxin which is readily detectable on polyacrylamide gels was found associated with the high density nucleocapsid and none was present in the vesicle fraction dissociated from the virions (Fig. 3). This finding can perhaps be attributed to charge interaction and association of the acidic pardaxin (6) with the basic M protein, which has a pI > 9.0 by isoelectric focusing (9). It should be noted that small amounts of M
The capacity of pardaxin to dissociate the VS virion membrane protein and pardaxin could have been present in other regions of the gradient that were not examined by polyacrylamide gel electrophoresis. These studies support the hypothesis (2, 4, 5, 10) that the M protein of both rhabdoviruses and paramyxoviruses serves as the “glue” that binds the nucleocapsid to the lipoprotein membrane of VS virus during the process of assembly and budding from the infected cell surface membrane. The M protein may have a greater affinity for the nucleocapsid than for the membrane as judged by its segregation during pardaxin-mediated fractionation of the two components of VS virions. A similar preferential association of M protein with nucleocapsids was found after VS virions were exposed to octylglucoside or Triton X-100 in the absence of salt (11).

An important question about the VS virion membrane is whether the intrinsic G protein traverses the bilayer and, if so, does the COOH terminus protrude from the inner surface? The capacity of pardaxin to dissociate the VS virion membrane, thus exposing the inner surface, seemed to provide a means to determine the interior location of the G protein. The primary amino acid sequence of the VS viral G protein is known from the studies of Rose et al. (12) who determined the nucleotide sequence of the cDNA copied from the G protein mRNA. According to their model, there is a hydrophobic domain of 20 amino acids extending from position 30 to 49 from the COOH terminus, ending in a COOH-terminal sequence of 29 hydrophilic amino acids. There is one tyrosine residue at position 11 from the COOH terminus; the next tyrosine is at position 111 (12). Therefore, it seems feasible by proteolytic digestion of the major external segment of the G protein, followed by selective iodination of hydrophobic tail segment, to determine whether the COOH-distal segment does indeed protrude beyond the inner membrane surface. For this purpose, VS virions were labeled with 125I by the chloramine T oxidation method as well as by the lactoperoxidase method (13) after digesting away the outer 90% of the G protein with thermolysin (3). Table II shows that the 66-amino acid G protein tail fragment generated by exposure of virions to thermolysin was specifically and heavily labeled with 125I in vesicles prepared from pardaxin-treated virions as determined by electrophoresis on a 17.5% sodium dodecyl sulfate-polyacrylamide gel (14). The G protein tail fragment of untreated virions was also labeled to some extent probably due to permeabilization and mechanical disruption during protease treatment of the virions. However, iodination of the tail fragment was 2-3-fold higher in the presence of pardaxin compared to untreated controls. These data suggest that the COOH terminus of the G protein protrudes from the inner surface of the VS virion bilayer at least as far as the tyrosine residue at position 11. It should be noted that there are also two histidine residues at positions 18 and 25 from the COOH terminus, both of which presumably protrude inward from the bilayer and could also be iodinated, but at lower efficiency.

Although detergents have served as useful tools for studying membrane dissolution and in vitro transcription of negative strand viruses, it is unlikely that this reaction mimics the

![Fig. 3. Polyacrylamide slab gel electrophoresis of proteins associated with vesicle (Ve) and nucleocapsid (NC) fractions of pardaxin-treated VS virions compared with proteins of whole virions (VSV). 300 μg of purified VS virions labeled with [3H]palmitic acid or [3H]uridine were allowed to react with 120 μg of pardaxin or with buffer (intact virions) and subjected to equilibration sedimentation by flotation through a 10-50% sucrose gradient as described in Fig. 2. Three fractions containing [3H]uridine label were collected from the bottom of the gradient and pooled, whereas fractions 19, 20, and 21 containing [3H]palmitate label were collected from the top of the other gradient and pooled. Intact virions were collected from fractions 9, 10, and 11 from a separate gradient. Densities of peak fractions were measured by refractometry. Fractions were dialyzed free of sucrose and lyophilized. Proteins from each sample were extracted by boiling in sodium dodecyl sulfate and were analyzed by electrophoresis on a Tris-glycine-buffered polyacrylamide slab gel consisting of a 2-cm stacking gel of 4% acrylamide and a resolving gel of 12.5% acrylamide. After fixation for 30 min in 50% methanol and 10% acetic acid, the gel was stained for 1 h with 0.1% Coomassie brilliant blue. L, G, N, NS, and M indicate the location of each viral protein. P designates the location of pardaxin (M, ~17,000) in the nucleocapsid fraction (NC) as determined by electrophoresis of the purified protein on a separate gel (not shown).]

### Table I

Comparative protein composition of high density nucleocapsids and low density membrane vesicles prepared from pardaxin-treated VS virions

| Virus preparation | Density | Viral proteins |
|-------------------|---------|----------------|
|                   | g/ml    | L  | G  | N  | NS | M   |
| Intact virions    | 1.16    | 11.7| 28.1| 25.3| 7.1 | 28.0|
| Nucleocapsids     | 1.20    | 10.7| 15.4| 41.4| 7.2 | 26.0|
| Vesicles          | 1.06    | <0.5| 69.3| 30.2| <0.5| <0.5|

### Table II

Iodination of the hydrophobic tail fragment of the VS viral G protein before or after exposure to pardaxin

A suspension of VS virions was treated with thermolysin (EC 3.4.24.4; Sigma; 30 units/mg of viral protein) for 30 min at 37 °C and then purified by centrifugation in a 10-40% sucrose density gradient. Control and pardaxin-treated spikeless virus was iodinated by chloramine T or by lactoperoxidase as described by Moore et al. (13). Iodinated samples were subsequently dialyzed overnight against phosphate-buffered saline (pH 7.4) and then subjected to 17.5% polyacrylamide gel electrophoresis to separate the hydrophobic piece of G protein (13). The gel fraction containing the stained G protein tail fragment was analyzed for 125I radioactivity by scintillation spectrometry.

| Mode of iodination     | Thermolysin alone | Thermolysin + pardaxin |
|------------------------|-------------------|------------------------|
| Lactoperoxidase        | 13.76             | 33.23                  |
| Chloramine T           | 16.90             | 42.20                  |

**TABLE I**

**Comparative protein composition of high density nucleocapsids and low density membrane vesicles prepared from pardaxin-treated VS virions**

The percentage of each viral protein (L, G, N, NS, and M) present in the intact virions, nucleocapsids, and vesicles of the electrophograms shown in Fig. 3 was determined by densitometry and integration.

**TABLE II**

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events that lead to dissociation of virion membrane and nucleocapsid when virus penetrates the host cell as infection is initiated. We have recently demonstrated that pardaxin at low doses can induce in vitro transcription of VS virions in the absence of detergents and at somewhat greater efficiency (15). The studies presented here suggest that pardaxin may provide a more physiological means for studying in vitro the dissociation of virion membrane and nucleocapsid that more closely resembles the intracellular events leading to initiation of infection.

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