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Resistance of the 64K Protein of Budded *Autographa californica* Nuclear Polyhedrosis Virus to Functional Inactivation by Proteolysis

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The 64K surface protein of budded *Autographa californica* nuclear polyhedrosis virus (AcMNPV BV) is known to play a role in the functional entry of AcMNPV BV into *Spodoptera frugiperda* IPLB-SF-21 cells by adsorptive endocytosis. AcV_1, a neutralizing monoclonal antibody, reacts with the 64K protein and in doing so prevents efficient entry. In this communication we report that treatment of AcMNPV BV with either trypsin or proteinase K cleaves the 64K protein into one major fragment of 34.6K and two minor fragments of 36K to 37.2K that are retained with the virus. All of the fragments are glycosylated. Protease treatment does not reduce viral infectivity, but it does result in the destruction of the AcV_1-reactive epitope; thus AcV_1 is not able to neutralize protease-treated AcMNPV BV. Polyclonal antiserum to BV is able to recognize both cleaved and uncleaved 64K and neutralize both protease-treated and untreated virus. Protease treatment does not diminish the sensitivity of AcMNPV BV to chloroquine, but it does cause the virus to become more susceptible to inactivation by 2-mercaptoethanol (2-ME) even though exposure to 2-ME does not result in dissociation of the fragments from the virus.

Autographa californica nuclear polyhedrosis virus (AcMNPV) is a member of subgroup A of the family Baculoviridae (*9*). Baculoviruses are restricted to arthropod hosts, and most infect lepidopteran insects. Baculoviruses of subgroups A and B have a unique survival strategy in that they use two phenotypes to complete an infection cycle in nature. One phenotype, made environmentally stable by its inclusion within a crystalline proteinic matrix, initiates infection in midgut cells after being ingested by a susceptible insect host. The other phenotype amplifies the infection systemically, and matures by budding from the plasma membrane of infected cells as enveloped single nucleocapsids. The budded virus (BV) differs from the occluded virus in many ways (*15*). One significant difference is that BV has a major surface antigen, a phosphoglycoprotein of 64,000 mol wt (64K) that functions in the efficient entry of BV by adsorptive endocytosis (*17, 18*). This protein is largely, if not totally, responsible for the significantly greater efficiency of infection of BV relative to the occluded form both in the insect hemocoel and in cell culture (*6, 14*).

In this study, we document the effects of trypsin and proteinase K on 64K of AcMNPV BV and compare protease-treated and untreated virus with regard to changes in infectivity after exposure to various biological and chemical reagents.

The cell lines used in this study, IPLB-SF-21 and TN-368, were derived from *Spodoptera frugiperda* and *Trichoplusia ni*, respectively (*16*). The TN-368 cells were grown in TNM-FH medium (*5*), and the IPLB-SF-21 cells were grown in TC-100 medium (*4*). The infectivity assay was the peroxidase antiperoxidase immunoassay described previously (*16*) with an incubation time of 40 hr, using IPLB-SF-21 cells unless specifically stated otherwise. BV was labeled with [^{35}S]methionine and harvested as reported previously (*18*). Routine protease treatment of BV consisted of incubation in 1 mg/ml proteinase K for 40 min at 4° or 1 mg/ml trypsin for 40 min at 27°, both in 0.05 *M* phosphate-buffered saline (PBS), pH 7.2, followed by addition of phenylmethylsulfonyl fluoride (PMSF) to 0.004 *M* and aprotinin to 2%. Protease-treated virus was then pelleted by centrifugation (50,000 *g* for 1 hr) through a 25% sucrose cushion and resuspended in PBS containing 0.002 *M* PMSF and 1% aprotinin. In order to visualize the 64K protein and its cleavage fragments clearly, radiolabeled virus was mixed with solubilization buffer (PBS containing 0.5% Nonidet-P40, 0.877% B-D octylglucoside, 1% aprotinin, and 0.002 *M* PMSF) at 27° overnight and pelleted by centrifugation at 50,000 *g* for 1 hr. The supernate of these samples was considered the soluble fraction and contained most of the 64K protein (or its cleavage fragments), and the pellet was considered the particulate fraction. These samples were analyzed by SDS–PAGE and autoradiography (*18*).

The effects of protease treatment on BV structural proteins are shown in Fig. 1. Analysis of the SDS–PAGE migration patterns of solubilized proteins, the nonsolubilized pelleted proteins, and the nonsolubi-

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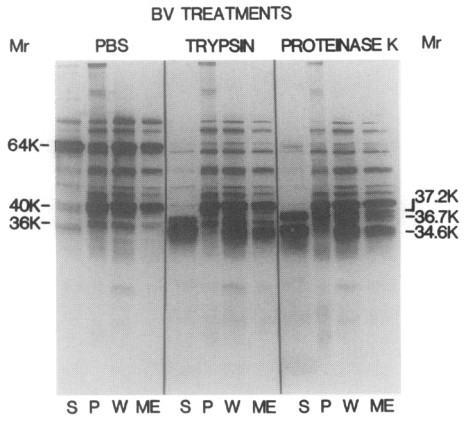

FIG. 1. Effect of protease treatment and exposure to 4% 2-ME on BV structural proteins. [^{35}S]methionine-labeled BV, treated with PBS alone or PBS containing trypsin or proteinase K, was pelleted through a 25% sucrose cushion, resuspended in PBS, and then analyzed by SDS–PAGE/autoradiography. Soluble (S) and particulate (P) fractions, as well as whole virus (W), were analyzed. Samples of protease-treated and untreated whole virus were additionally incubated with 4% 2-ME at 37° for 15 min before analysis (ME).

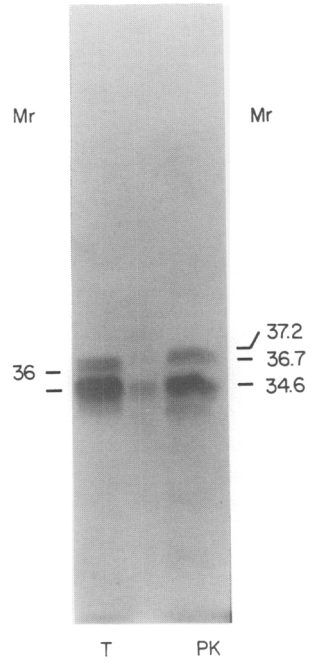

FIG. 2. Protease cleavage of *N*-acetyl-D-[1-^{3}H]glucosamine-labeled 64K. *N*-acetyl-D-[1-^{3}H]glucosamine-labeled BV was treated with PBS containing trypsin (T) or proteinase K (PK) before being solubilized and the soluble fractions were analyzed by SDS–PAGE/autoradiography.

lized virus reveals that only the 64K protein appeared to be cleaved by the protease treatments. The major cleavage product of both proteinase K and trypsin was a fragment of estimated mol wt 34.6K. The molecular weights of the minor tryptic products were estimated to be 36K and 36.7K, while the minor proteinase K products were 36.7K and 37.2K. It is interesting that trypsin and proteinase K, quite different in their cleavage site specificities (*1, 2*), cleaved 64K into such similar patterns. Surprisingly, the infectivity of protease-treated BV was not significantly altered from that of the starting BV preparation, as assessed both by plaque assay in cell culture and by intrahemocoelic injection into mid-fourth instar *T. ni* larvae (*6*), suggesting that the cleavage fragments of 64K retained by the virus after protease treatment contained the biologically active portions of the protein (data not shown).

It is known that the 64K protein of BV is a glycoprotein (*18*). To determine which of the protease-generated cleavage fragments were glycosylated we labeled BV with *N*-acetyl-D-[1-^{3}-H]glucosamine as described before and treated it with protease, and examined the soluble fraction by SDS–PAGE and autoradiography. Figure 2 shows that for both trypsin and proteinase K-treated BV, all the fragments were glycosylated. It is probable that the carbohydrate moiety of the fragments protests them somewhat from proteolytic degradation, which would explain the similarity of the fragment patterns generated by the two different proteases.

BV and protease-treated BV were exposed to various concentrations of 2-mercaptoethanol (2-ME) to determine the effect on infectivity. These experiments were done by incubating aliquots of virus in PBS containing 0.002 *M* PMSF, 1% aprotinin, and 0–4% 2-ME for 15 min at 37°, pelleting the virus through a 25% sucrose cushion by centrifugation at 50,000 *g* for 60 min, resuspending the samples in medium, and assaying for infectivity. The results, seen in Table 1, indicate that while the infectivity of all three preparations was affected, protease-treated BV was more sensitive than

TABLE 1

COMPARATIVE EFFECTS OF 2-ME ON THE INFECTIVITY OF PROTEASE-TREATED AND UNTREATED BV

	BV treatments		
% 2-ME[a]	PBS	Trypsin	Proteinase K
0	5.90[b] (0)[c]	6.30 (0)	6.41 (0)
1.0	5.88 (0.02)	5.78 (0.52)	6.10 (0.31)
2.0	5.70 (0.20)	3.30 (3.00)	4.71 (2.24)
4.0	2.48 (3.42)	3.16 (3.14)	2.70 (3.71)

[a] Virus was incubated with 2-ME at the indicated concentrations for 15 min at 37° before being assayed for infectivity.

[b] The mean titer (PFU/ml) is expressed as the base 10 logarithm. Standard deviations were all within 0.5 $\log_{10}$ units of the mean.

[c] The number in parentheses is the decrease in titer from the 0% 2-ME value.

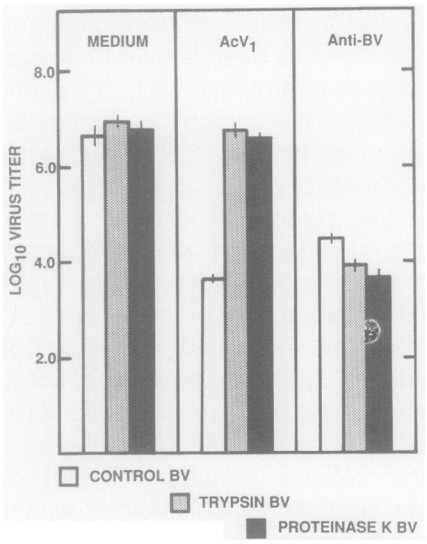

FIG. 3. Neutralization of BV and protease-treated BV with AcV_1 and anti-BV. BV, trypsin-treated BV, and proteinase K-treated BV were incubated with medium, medium containing AcV_1, or medium containing anti-BV for 1 hr at 37° before being assayed for infectivity.

BV. Both trypsin and proteinase K-treated virus lost significant infectivity (over 100-fold) after exposure to 2% 2-ME, while the PBS-treated control preparation lost minimal infectivity. The infectivity of all three preparations decreased over 1000-fold after exposure to 4% 2-ME, however. In an effort to determine whether this loss of infectivity was due to dissociation of 64K or its cleavage fragments from the virus, [^{35}S]methionine-labeled BV and protease-treated BV were analyzed by SDS–PAGE and autoradiography following exposure to 4% 2-ME for 15 min at 37°. Neither 64K nor its proteolytic cleavage fragments were dissociated from the virus (Fig. 1, lanes marked ME). These results indicate that while neither 64K nor its cleavage fragments appear to be thiol linked to virus, disulfide bonds are important in the conformation of their biologically active domain(s). The increased sensitivity of the retained cleavage fragments to 2-ME could be explained if the fragments contained fewer disulfide bonds than the uncleaved protein, and/or if protease cleavage caused the disulfide bonds to become more accessible.

In order to determine whether protease-treated BV could be neutralized by AcV_1 and anti-BV, neutralization experiments were performed by incubating protease-treated and untreated BV for 1 hr at 37° in either a 1:42 dilution (in medium) of rabbit antiserum to BV (anti-BV) (*13*) or 0.12 μg/ml medium of affinity-purified AcV_1 monoclonal antibody (*17*). Following the incubation period, the infectivity of the samples was determined. The results were that BV was significantly neutralized by both antibody preparations, but protease-treated BV was neutralized only by antiserum to BV (Fig. 3). To ensure that the lack of AcV_1 neutralizing activity for protease-treated BV was not due to antibody degradation by residual protease, we tested for the presence of active AcV_1 in the AcV_1–protease-treated BV neutralization samples. After incubation, the samples were centrifuged at 100,000 *g* to remove most of the protease-treated BV, then the supernatant fluids were tested for residual infectivity and for their ability to neutralize freshly added BV. The BV neutralizing capacity was found to be intact; it reduced the infectivity of the BV-spiked preparations by 2.7 orders of magnitude (data not shown).

To determine whether the protease-generated fragments of 64K reacted with AcV_1 and anti-BV, immunoprecipitation experiments were done as described previously—except this time we used a higher concentration of PMSF (0.004 *M*). The results (Fig. 4) indicated that while anti-BV recognized 64K as well as all the cleavage fragments generated by both trypsin and proteinase K, AcV_1 recognized only the uncleaved 64K. The AcV_1-reactive epitope apparently was destroyed by the protease treatments, which accounts for the lack of AcV_1 neutralizing activity against protease-treated BV. The fact that particles lacking the AcV_1-reactive epitope were no less infectious than normal particles indicates that even though antibody binding of the epitope neutralizes the virus, the epitope itself is not important for infection.

Previous studies have shown that populations of BV saturated with AcV_1 are reduced in titer 1000- to 10,000-fold, but that complete neutralization does not occur (*18*). Studies have shown that the residual infectivity of AcV_1-saturated BV, unlike BV, is not sensitive

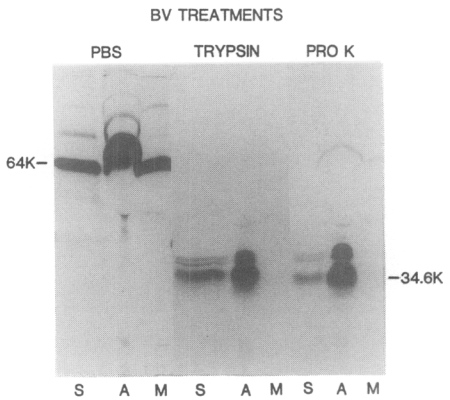

FIG. 4. Immunoprecipitation of 64K and its cleavage fragments by AcV_1 and anti-BV. Solubilized (S) [^{35}S]methionine-labeled 64K or protease-generated cleavage fragments of 64K were incubated with anti-BV (A, antiserum) or AcV_1 (M, monoclonal), immunoprecipitated with protein A–Sepharose CI-4B beads, and analyzed by SDS–PAGE and autoradiography.

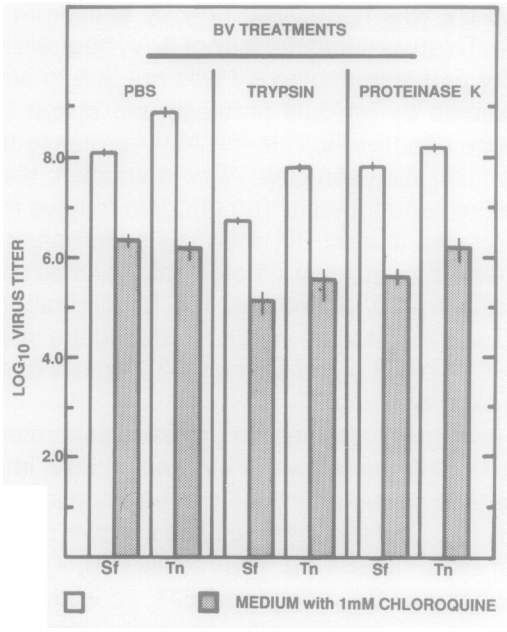

FIG. 5. Chloroquine-sensitivity of BV and protease-treated BV in IPLB-SF-21 (SF) and TN-368 (Tn) cells. The infectivity of BV and protease-treated BV was compared in the presence and absence of 1 m*M* chloroquine.

to inhibition by lipophilic amines. These studies resulted in the hypothesis that the function of the 64K protein is to allow efficient entry of BV by adsorptive endocytosis. Because proteolytic cleavage renders BV nonneutralizable by AcV_1 but does not reduce infectivity, it occurred to us that such a cleavage of 64K might be a normal part of the infection process after adsorption of BV to susceptible host cells. Natural proteolytic cleavage of surface glycoproteins has been shown for several enveloped viruses, including paramyxo, myxo, and coronaviruses (*3, 7, 8, 10–12*). This cleavage frequently plays an important role in viral infectivity and host specificity. If such a cleavage takes place in the course of AcMNPV BV infection, we reasoned that it might take place in and be dependent on the acidic environment of endocytic vesicles. We were interested, therefore, in determining whether protease-treated BV infectivitiy was sensitive to inhibition by chloroquine, a lipophilic amine that prevents the acidification of endosomes. This was done by performing infectivity assays in the presence of 1 m*M* chloroquine as described previously (*17*). The results in Fig. 5 show that infection of both IPLB-SF-21 and TN-368 cells by protease-treated BV is sensitive to chloroquine. Cleavage of 64K does not release BV from the requirement of functionally entering cells via acidic endosomes. If protease cleavage does take place in a natural infection and is dependent on an acidic environment, it is not the only event important to BV infectivitiy that is pH-sensitive and takes place in endosomes.

The infectivitiy of some of the viruses requiring proteolytic cleavage has been shown to be suppressable by the presence of protease inhibitors during infection (*3, 19, 20*). As part of a search for evidence of a required natural proteolytic cleavage of 64K during infection, we tested the effects of various protease inhibitors on viral infectivity. We incubated cells for 2 hr before and throughout infection with various concentrations of leupeptin, aprotinin, pepstatin, *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), and 6-amino-*n*-hexanoic acid but found no evidence that any one of them adversely affected BV infectivity (data not shown).

Another approach taken in search for a role for natural proteolytic cleavage was to look for an increase in infectivity of protease-treated over that of untreated BV in alternate, semipermissive cell lines, with the hypothesis that their low susceptibility could be due to the lack of necessary proteolytic enzymes. In a previous study we documented the relative susceptibilities of several cell lines to AcMNPV BV infection (*16*). Two of the least susceptible were MRRL-CH and IPLB-SD-652, cell lines derived from *Manduca sexta* and *Lymantria dispar,* respectively. The infectivities of protease-treated and untreated BV were compared using these two cell lines with the result that protease treatment did not induce greater infectivity, but rather slightly less (data not shown).

Although the approaches taken to generate evidence that protease cleavage of 64K might be a natural and necessary part of infection were preliminary and by no means exhaustive, the lack of such evidence led to an alternate hypothesis that we might be observing resistance to functional inactivation by proteases instead of activation. We reasoned it was possible that BV could be exposed to proteases during natural infection in the insect hemocoel, and if so, had to be somewhat functionally resistant to proteolysis in order to remain infectious.

To test this possibility, hemolymph was removed from several fourth instar *T. ni* larvae infected for 48 and 96 hr. The larvae were each infected by *per os* injection of 1 ng of polyhedra-derived AcMNPV. This dose represents about 666 LD_{50} doses (*6*). The hemolymph was diluted in medium and the titer of the virus and its sensitivity to neutralization by AcV_1 and anti-BV was determined as before (Fig. 3). The results were that hemolymph-derived virus from both 48- and 96 hr-infected larvae was neutralized by both AcV_1 and anti-BV (data not shown). These results indicated that the virus was not subjected to severe proteolysis in the hemolymph even by 96 hr postinfection when obvious signs of disease were apparent.

Our search for a biological explanation for the observed functional resistance of the 64K protein to pro-

teolytic degradation was unsuccessful. Nevertheless we found that the biological activity of 64K was contained in fragments about half the size of the native protein. The fragments were glycosylated and located proximal to the virus surface relative to the portion that is degraded by proteases. We found that disulfide bonds play an important role in maintaining the conformation of biologically important domains of both native and truncated 64K, but do not serve to link them to the virion. Finally, we found that the epitope reactive to neutralizing antibody AcV_1 does not play an important role in BV infectivity.

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