Immunoabsorbent nanoparticles based on a tobamovirus displaying protein A

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Earlier attempts to express peptides longer than 20 aa on the surface of tobamoviruses such as tobacco mosaic virus have failed. Surprisingly, we found that a functional fragment of protein A (133 aa) can be displayed on the surface of a tobamovirus as a C-terminal fusion to the coat protein via a 15-aa linker. The macromolecular nature of these nanoparticles allowed the design of a simple protocol for purification of mAbs with a recovery yield of 50% and >90% product purity. The extremely dense packing of protein A on the nanoparticles (>2,100 copies per viral particle) results in an immunoabsorbent with a binding capacity of 2 g mAb per g. This characteristic, combined with the high level of expression of the nanoparticles (>3 g adsorbent per kg of leaf biomass), provides a very inexpensive self-assembling matrix that could meet the criteria for a single-use industrial immunoabsorbent for antibody purification.

antibody purification | coat protein fusion | viral particle

Because of their relatively simple macromolecular organization and very high accumulation titers, plant tobamoviruses provide an extremely cheap source of protein biopolymers of discrete nanoparticle sizes than can be manufactured rapidly and under very simple conditions. Tobacco mosaic virus (TMV), the best-known member of the tobamoviruses, is also one of the most extensively studied viruses (1). It has a positive-sense RNA genome encoded in a single 6.4-kb RNA molecule. The genome encodes four proteins, including the 17.5-kDa coat protein (CP), the most abundant viral product and the only component of the TMV capsid. More than 2,100 copies of CP fully protect the single-stranded viral RNA, resulting in rigid rod-shaped viral particles 300 nm in length and 18 nm in diameter, with a molecular mass of 40,000 kDa. Considering that TMV accumulates to levels of up to 10 g per kg of leaf biomass and that CP accounts for 95% of the mass of the viral particles, the CP therefore represents the most abundant protein that can be harvested from plants. The viral particles can be purified industrially by using simple “low-tech” protocols (1, 2). Because of the ability of the CP to polymerize *in vivo* and *in vitro* and the high stability and defined size of the assembled virions, the CP represents a potentially promising biopolymer feedstock for a number of applications in nanobiotechnology.

The CP-based polymer can be modified in two different ways. One approach consists of adding novel moieties to viral particles *in vitro* by chemical modification (3, 4). The other avenue consists of genetically reprogramming the virus to express protein fusions, thus allowing the addition of new functional blocks to the viral core monomer, the CP (5). Because the structure of the TMV particle has been determined at atomic resolution (6, 7), fusion proteins can be designed in such a way that the added sequences are predicted to lie on the surface of the assembled viral particle. Despite this knowledge, attempts by numerous groups to create new products based on protein fusions have so far met with limited success. CP fusion proteins carrying an up to 20-aa extension to the C-terminus or a 12-aa insertion in a surface loop of the CP were shown to assemble into infectious virions, with the displayed peptides ranging from neuropeptides (5, 8), to vaccine epitopes (9–11), to affinity tags

(12). However, addition of larger peptides at the C-terminus of TMV CP always proved to be assembly-defective.

We have decided to reexamine the issue of the size constraint on proteins that can be fused to the CP of tobamoviruses without preventing viral particle formation. We have explored approaches taken by others who have designed systems for display of foreign proteins on the surface of filamentous bacteriophages (13). Among other parameters, we have tested the effect of short linkers introduced between the two fusion components on the functionality of both fusion partners (14, 15). We report here data on the fusion of the CP of turnip vein clearing virus (TVCV) with a functional fragment of protein A from *Staphylococcus aureus*. Protein A is an antibody-binding agent that is widely used for industrial-scale purification of IgG antibodies from different sources (16, 17). We found that a 133-aa fragment of protein A that contains two of the five Ig binding domains can be displayed on the surface of TVCV as a C-terminal fusion to CP via a short flexible linker. The resultant chimera retains the ability to assemble into viral particles, whereas the protein A fragment remains fully functional as an immunoabsorbent that can be used for purification of monoclonal antibodies.

Results

CP-Protein A Fusion Is Expressed at a High Level in Plant Tissue Using Viral Vectors. TVCV CP fused to a protein A fragment (domains E and D) was expressed in *Nicotiana benthamiana* leaves by using the proviral vector system described earlier (18). The CP and protein A domains were cloned separately in 5'- and 3'-provector modules, and these modules assembled into a complete vector by site-specific recombination, *in planta*, after *Agrobacterium*-mediated delivery into plant cells. The protein A moiety was fused either N- or C-terminally, with or without a 15-aa linker peptide (Fig. 1A). Two different linkers were tested: a flexible glycine-rich linker, (GGGGS)₃, and a helical linker, (EAAAK)₃. Both N- and C-terminal fusion proteins containing either linker were expressed at high level and could be detected on Coomassie-stained gels as a strong band comparable to that of wild-type CP (Fig. 1B). In contrast, fusion proteins without a linker were not expressed at detectable levels.

Surprisingly, although plants were inoculated by infiltration of *Agrobacterium* on the lower leaves only, viral disease symptoms could be seen in the upper noninfiltrated leaves. Analysis of the crude extract prepared from these leaves revealed that they contained high level of the fusion protein (Fig. 1B), suggesting that the presence of protein A does not prevent the formation of viral particles and does not prevent systemic movement of the virus.

CP-Protein A Fusion Assembles into Functional Viral Particles. To test whether viral particles were actually made, we used standard

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Abbreviations: TMV, tobacco mosaic virus; CP, coat protein; TVCV, turnip vein clearing virus.

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Fig. 1. High-level expression of protein A-viral particles. (A) Constructs used for transfection of *N. benthamiana* plants. Wild-type CP is expressed from an assembled vector (pICH17501). CP-protein A fusions are expressed from separate 5' and 3' modules that are assembled *in planta* by using a site-specific recombinase (pICH10881). Short 15-aa linkers (hatched boxes) are included in the 5' modules (except for pICH20697): a flexible linker (GGGG)₃ in pICH20701 and pICH24384 and a helical linker (EAAAK)₃ in pICH20723 and pICH24399. White boxes represent introns. RdRp, RNA-dependent RNA polymerase; MP, movement protein; attP/attB, recombination sites; int, 5' and 3' part of intron for removal of the recombination site through splicing; N, 3' nontranslated region; T, NOS terminator; LB and RB, T-DNA left and right borders; *, linker absent in pICH20697. (B) Coomassie-stained polyacrylamide gel showing crude extracts from plants transfected with wild-type virus, protein A N-terminal fusions with linkers (PA-CP), protein A C-terminal fusions (CP-PA) without (pICH20697) or with (pICH20701/20723) linkers, C-terminal fusion with linker, systemic leaf (CP-PA sys), and nontransfected control. Equal amounts of crude extracts were loaded. Rbc, Rubisco large subunit.

procedures for the isolation of such particles. Analysis of the purified samples revealed that CP-fusion proteins could be recovered at high yield (≈ 3 g per kg of leaf biomass), but only when protein A was fused to the C-terminus of CP. As shown on a Coomassie-stained gel (Fig. 2A, data shown only for the glycine-rich linker although similar results were also obtained with the helical linker), the purified samples contained a major protein band of the expected size for the fusion protein (33.3 kDa) and for the wild-type control (17.5 kDa). For the fusion protein samples, a few minor bands (between 17.5 and 33.3 kDa) could also be seen, suggesting either some instability of the construct during replication or partial degradation of the protein fusion before or during purification (see below). Analysis of the purified protein by peptide mass fingerprinting indicated that,

except for the N-terminal methionine, the fusion protein is completely synthesized and recovered after purification (data not shown).

Further analysis of the purified samples with a transmission electron microscope confirmed that particles were indeed made (Fig. 2B and C). The length of the chimeric particles was similar to that of the wild-type particles (≈ 200 – 220 nm length). However, the chimeric particles differed from the wild type by adsorption of more staining solution on the sides of the particles, resulting in a higher electron density surrounding the particles. This difference was most obvious in mixed preparations of wild type and chimera (Fig. 3A). These results indicate that the protein A moiety forms a loosely condensed layer that is easily accessible to solvent and other proteins (e.g., immunoglobulins, see below).

Fig. 2. Purification of viral particles. (A) Coomassie-stained gel showing different steps of purification of the protein A chimeric particles (pICH20701-pICH21767) and the wild-type virus. Crude extracts (1), chloroform-extracted samples (2), resuspended pellet after first PEG precipitation (3), and resuspended pellet after second PEG precipitation (4). (B–D) Electron micrographs of viral particles. Protein A C-terminal fusion (pICH20701-pICH21767), purified (B), wild type (pICH17501), purified (C), and protein A N-terminal fusion (pICH24384-pICH23939), crude extract (D). (Scale bars: 100 nm.) (E) Stability of protein A virions (pICH20701-pICH21767). Samples were stored for 2 weeks at 4°C, at –20°C or at 4°C including protease inhibitors.

Fig. 3. Binding and precipitation of immunoglobulins by using protein A particles (pICH20701-pICH21767). (A) Immunogold labeling of a mixed sample of protein A- and wild-type virions. Gold-conjugated antibodies are only bound to the protein A particle that is easily recognized by its electron-dense appearance. (Scale bar: 100 nm.) (B) Coomassie-stained gels demonstrating the binding capacity of the protein A nanoparticles. A constant amount of particles was mixed with increasing amounts of human immunoglobulins and precipitated as described in *Methods*. Supernatants (s) and resuspended pellets (p) were analyzed by SDS/PAGE. Controls include samples without virus and with wild-type virus. HC, IgG heavy chain; LC, IgG light chain.

To determine whether the protein A fusions at the N-terminus of CP also assemble into viral particles, crude extracts, rather than purified samples, were analyzed by using the electron microscope. Surprisingly, only large aggregates of viral particles were observed (Fig. 2D). Considering that we never observed similar aggregation in crude extracts from C-terminal fusions (data not shown), it appears likely that aggregation of the N-terminal fusions is the cause for the loss of the particles during the purification procedure.

As mentioned above, the purified CP-protein A particles appeared to show signs of degradation. Therefore, we analyzed the stability of the purified particles under different storage conditions. Whereas wild-type particles were very stable at 4°C (data not shown), we detected a strong decrease in size for the fusion protein within several days at the same temperature (Fig. 2E). Interestingly, a protein slightly larger than the wild-type CP accumulated as a final breakdown product, suggesting that only the protein A part exposed at the surface of the particle is degraded. Degradation was prevented by storage at −20°C or by adding protease inhibitors (2 mM EDTA and 1 mM PMSF) to the storage buffer.

Protein A-Coated Particles Bind Antibodies Efficiently. Binding of antibodies to the protein A moiety was first demonstrated on purified particles by using immunogold labeling. Because protein A binds efficiently to the constant regions of many subclasses of IgG from different species (19), this test could be done either directly by using any gold-conjugated antibody or indirectly by using a primary unlabeled and a secondary gold-conjugated antibody. Although the first approach already gave good results (data not shown), these were further improved by including a secondary antibody. In a mixture of wild-type and protein A particles, only the latter were clearly labeled (Fig. 3A). We obtained similar results by blotting the CPs onto PVDF membrane and probing with a peroxidase-conjugated antibody. We detected signals only for the fusion protein (see also Fig. 5B) and not for the wild-type CP.

The binding capacity of the protein A-coated particles for IgGs was quantified by using a commercial preparation of human IgGs purified from serum. IgGs were bound and precipitated up to ≈2 g per gram of protein A particles under optimal conditions, whereas they did not bind at all to wild-type particles (Fig. 3B). Considering the molecular weights of CP-protein A (33 kDa) and of IgGs (≈155 kDa), we calculated that one IgG molecule is bound to every third to fifth subunit of the particle. As for the experiments illustrated in Fig. 2, the experiments shown in Fig. 3 (the immunogold labeling and IgG binding assay) were also performed by using both linkers, but the results are only shown for the glycine-rich linker. Because protein fusions with either linker again did not behave differently, we decided to focus further experiments on the particles with the glycine-rich linker.

As a next step, we sought for a strategy to separate the IgGs

from the particles. We found that the easiest way was to resuspend the precipitate in a buffer favoring dissociation (0.1 M glycine, pH 2.5) and then to pellet the viral particles by addition of PEG (4% [wt/vol]) as described for the purification of particles. The IgGs are left in the supernatant and are recovered by a further increase in the PEG concentration to 15% (wt/vol), leading to their precipitation. Although part of the IgGs was lost in the first step, most of the remaining IgGs could be recovered in a highly purified form, with an overall yield of ≈50% (Fig. 4).

To compare our purification protocol with established standard methods, we used protein A magnetic beads to perform the same experiment. Analysis of these samples revealed that, at this scale, the protein A viral particles were equivalent to a standard antibody purification agent (Fig. 4).

Antibodies Expressed in Plants Can Be Purified By Using Protein A-Coated Particles. The production of monoclonal antibodies (mAbs) in plants represents an increasing field of research in both academia and the biotechnology industry (20). We therefore wanted to know whether the protein A nanoparticles would also be useful for purification of antibodies from such material. We used monoclonal antibodies that were expressed in *N.*

Fig. 4. Efficient binding and release of immunoglobulins by using protein A nanoparticles (pICH20701-pICH21767). Samples from different steps were separated by SDS/PAGE, stained with Coomassie dye, or blotted onto PVDF membrane followed by detection of the heavy chain with a specific antibody. Samples include supernatant and precipitate of the particle-IgG complexes, removal of the viral particles by PEG precipitation and the purified IgG fraction. The last two lanes show the supernatant and eluate of a control purification using commercially available protein A magnetic beads. HC, IgG heavy chain; LC, IgG light chain.

Fig. 5. Protein A nanoparticles (pICH20701-pICH21767) can be used for purification of plant-made mAbs. (A) Coomassie (Left) and silver-stained (Right) gels, samples from different steps of the purification procedure as described in Fig. 4. Numbers in parentheses give the concentration factor relative to the crude extract. (B) Western blot with the samples described above. IgG heavy chain (HC) was detected with a specific antibody. Because of its general binding ability for immunoglobulins, CP-protein A is also detected. (C) Use of crude extract of virus-infected plant (right side of C) compared with pure nanoparticles (left side of C) for the purification of plant-made mAbs. The gel shows the supernatants and the resuspended pellets obtained after addition of protein A-virus and the purified mAbs. LC, IgG light chain.

benthamiana using technology developed at Icon Genetics (21). Viral particles ($\approx 100 \mu\text{g/ml}$) were added to extracts prepared from expressing plants, and the viral particle/mAb complexes were precipitated by centrifugation. The precipitate, which contained mostly CP-protein A and IgGs (Fig. 5A), was processed as described above. As estimated from a Western blot (Fig. 5B), $\approx 20\%$ of the expressed antibody heavy chains could be recovered by using this procedure. A similar result was obtained with a second antibody. The recovery yield for fully assembled mAbs is in fact probably $>20\%$, because the heavy chains that are detected in the crude extract may not necessarily be all assembled into functional antibodies, but only those that are assembled will be effectively purified using protein A. The purified antibodies retained full functionality, judging by binding to their antigen by using ELISA (data not shown).

In the specific application in which antibodies and viral particles are both expressed in plants, purifying viral particles to a high level of purity only to mix them later to a crude plant extract might be superfluous. Rather, simply using a crude preparation of viral particles might be sufficient and work just as well. Thus, leaves expressing viral particles were extracted and filtered as described in *Methods*. The filtrate was further clarified by a simple centrifugation step. This step did not reduce the amount of recovered particles (data not shown) but removed some insoluble material that could potentially interfere with IgG binding. This crude virus preparation was used in the same way as the pure particles and was also successful in purifying antibodies from plant tissue, although with slightly lower efficiency (Fig. 5C).

Discussion

Until now, only peptides shorter than 20 aa could be fused to the CP of plant rod-shape viruses without preventing assembly of functional virions (5, 22). The fusion of GFP to potato virus X CP has been reported to be able to assemble into viral particles, but only when free CP was coexpressed together with the fusion protein (23). In some cases, even the fusion of short peptides is enough to prevent virion formation (24). In this specific example, viral particles could form only when free CP was coexpressed together with the fusion protein (by introduction of a “leaky” stop codon between CP and the peptide), leading to the formation of viral particles displaying the peptide on $\approx 5\%$ of the CP subunits.

In contrast, our work clearly shows that large protein fragments (almost as large as the CP itself) can be successfully expressed as CP fusion and assembled into virions, even in the absence of free CP.

We found that addition of a 15-aa flexible or helical linker peptide between the CP and the fusion protein plays a pivotal role for correct folding of the protein fusion and particle assembly. This finding extends the utility of tobamovirus-based CP fusions to other important applications such as the manufacturing of affinity adsorbents and, potentially, of other biological nanopolymers.

Purification of pharmaceutical and other high-value proteins is an ongoing concern for biotechnologists, because commercial downstream processes used today are extremely complex and costly, accounting for as much as 50–80% of total manufacturing costs (25). In particular, the cost of protein A-based immuno-adsorbent, a standard reagent used for industrial purification of monoclonal antibodies, is so high that the columns have to be reused up to 50 times, resulting in significant costs due to multiple cleanings and revalidation of the columns. An ideal alternative has to be a single-use reagent that is cheap enough to provide sufficient economic advantages.

Several potential biotechnological solutions have been described in the literature that propose fusing protein A (or other affinity ligands) to elements capable of polymerizing (bacteriophage capsid proteins [26–28]) or binding to structures of high molecular weight (bacterial S-layer proteins [29, 30], oleosins recognizing oil bodies [31, 32], cellulose-binding domains [33, 34], starch-binding domains [35]). So far, only the process based on oleosin fusions (to protein A) has been advanced to the stage of a commercially viable platform (www.sembiosys.com). One limitation of this technology is the relatively low yield of oleosin fusion molecules ($\approx 1 \text{ kg}$ or less per hectare of safflower planting). The productivity of the virus-protein A system described here is significantly higher (calculated at 200–500 kg per hectare in an open field and up to 5 ton per hectare in a greenhouse), thus allowing for much cheaper manufacturing of the immuno-adsorbent, a more controlled production (greenhouse), and a smaller space required. The low cost of the immuno-adsorbent also means that it may have a future as a single-use (disposable) industrial reagent.

The simple mAb purification protocol developed in this study is robust and involves only low-tech components/reagents, such as low-speed centrifugation, polyethylene glycol, ionic strength, and pH of the buffer. The efficiency of the protocol in terms of yield and purity is equivalent to standard methods such as those relying on the use of protein A-columns or protein A coupled to magnetic or agarose beads (36). The use of a plant-derived immuno-adsorbent makes it an especially attractive tool for the purification of plant-made monoclonal

antibodies because it can be used in its crude form and does not require extensive processing. The binding capacity of the current CP-protein A (2 g of IgG per 1 g immunoabsorbent or one IgG molecule per 3–5 moieties of protein A) is 200-fold higher than that of the existing protein A carriers (30, 37).

For industrial applications, future protocols might be developed (based on the same or similar immunoabsorbent polymers) that more fully use the nanoparticle nature of the immunoabsorbent. Using coexpression techniques, we have designed TVCV particles that display, in addition to protein A, minor components of different functionality, including affinity tags and fluorescent markers. Preliminary data obtained in our laboratory indicate that after coexpression of two different CP fusions (i.e., CP-protein A and CP-Strep tag), both types of CP could be copurified by using a standard purification protocol, and both were coprecipitated after addition of IgGs, suggesting that the second CP fusion protein was indeed incorporated into the particles. This finding shows that it is possible to engineer additional ligands on the surface of the polymer, allowing the development of protocols for purification based on partitioning, e.g., by ligand-based precipitation/capture of the immunoabsorbent.

Industrial use of fully assembled plant virions as nanoparticle reagents also faces some problems that need to be addressed in future research. The polymer is in fact a self-reproducing organism, and although it is a ubiquitous plant virus and thus poses little or no risk to humans, it may require the development of some measures of control and containment. One possible solution would be the use of virus-inactivating chemicals like formaldehyde or binary ethyleneimine (38). Preliminary data in our laboratory indeed suggest that binary ethyleneimine treatment effectively inactivates the protein A-coated viral particles and that the immunoabsorbent properties of the particles are not affected. Other more sophisticated solutions would include the production of particles using technologies that do not require full functionality of the virions, such as magnification (39). In separate experiments, we (C. Engler, S.W., S.M., and Y.G., unpublished work) and, earlier, others (40, 41) have demonstrated that viral replicons and vectors lacking a functional movement protein gene (required for cell-to-cell movement of virus) can be fully complemented by transgenic plant hosts expressing the viral movement protein. Provided that the replicons contain the viral origin of assembly, the particles produced will be non-infectious on wild-type plants because they will lack a movement protein gene. In the future, the viral vectors could also be modified via multiple mutations that would render the virus conditionally viable or would block essential functions necessary for infectivity and/or survival of the virus in the open environment (42). The ultimate solution might be a polymer that is capable of self-assembly but does not package the viral RNA.

Methods

Constructs. Viral expression vectors used in this study are based on the TMV-related viruses TVCV (43) and CrTMV (44). The basic principle of proviral expression modules and their optimization for high-level expression was described (18, 39). The sequence of the IgG-binding domains E and D from the *S. aureus* protein A along with short sequences on both sides (amino acids 29–161; GenBank accession no. J01786) were optimized for expression in *Nicotiana* sp. and synthesized by Geneart (Regensburg, Germany). The gene was cloned into a standard 3' provector (construct pICH21767) and into 5' provectors containing 15-aa linker peptides consisting of three repeats of the sequence Gly-Gly-Gly-Gly-Ser (construct pICH24384) or Glu-Ala-Ala-Ala-Lys (construct pICH24399). pICH20697, pICH20701, and pICH20723 are 5' provectors containing the CP of TVCV without a stop codon. The C terminus of CP in pICH20701 and pICH20723 is fused to the linkers described

above. CP was also cloned into a 3' provector, resulting in construct pICH23939. Constructs were made by using PCR amplification with primers containing appropriate restriction sites for the subsequent cloning steps. The construct containing the phage C31 integrase (pICH10881) for *in planta* assembly of the viral modules was described in ref. 18. pICH17501 is a fully assembled viral vector containing the native CP.

Expression and Purification of Virus Particles. Transfection of *N. benthamiana* plants for expression of viral constructs was done as described (18). Infiltrated (or in some cases systemic) leaves were harvested 8–10 days postinoculation and homogenized with 2 ml 0.1 M potassium phosphate buffer (pH 7.0) per gram of fresh weight by using a leaf juice press (MEKU GmbH, Wennigsen, Germany). The homogenate was filtered through filter paper and extracted once with 0.25 volumes of chloroform. Viral particles were precipitated from the aqueous phase with 4% (wt/vol) PEG 6000 and 1% (wt/vol) NaCl. The precipitate was resuspended in 1/5 volume 0.01 M potassium phosphate buffer (pH 7.0). Insoluble material was removed by mild centrifugation (5 min, 5,000 × g, 4°C), and the PEG precipitation was repeated once more.

Binding and Purification of Immunoglobulins. IgGs purified from human serum were purchased from Sigma (St. Louis, MO). Plant-made monoclonal antibodies were expressed as recently described (21). We used two different antibodies for this study, both of IgG1 subtype (21). Plant material was homogenized in liquid N₂, suspended in 3 ml PBS (45) per gram of fresh weight, and insoluble material was removed by two rounds of centrifugation (10 min, 16,000 × g, 4°C). Viral particles decorated with protein A were added to the starting material (100 µg/ml) and incubated on ice for 30 min. Virus-IgG complexes were precipitated by centrifugation (10 min, 12,000 × g, 4°C) and resuspended in 0.5 volumes of 0.1 M glycine (pH 2.5) to dissociate the IgGs from the protein A. Viral particles were removed by PEG precipitation as described above. The supernatant containing the IgGs was neutralized with 1/10 volume 1 M Tris-HCl (pH 9.0) and mixed with an equal volume of 25% (wt/vol) PEG 6000 (dissolved in PBS; final PEG concentration of 14.5%). Samples were incubated 30–60 min on ice and IgGs were sedimented by centrifugation (15 min, 16,000 × g, 4°C). The supernatant was carefully removed and the IgGs were dissolved in a convenient volume of PBS.

Protein A magnetic beads were purchased from New England Biolabs (Beverly, MA) and used according to the manual.

Protein Analysis. Protein concentration was determined by using Bradford assay. Samples were separated by SDS/PAGE and blotted onto PVDF membranes by using standard procedures and equipment (BioRad, Hercules, CA). Blocking and detection were also done by standard procedures by using peroxidase-coupled antibodies against human IgG γ-chain (Sigma) and the ECL chemiluminescent detection reagent (Amersham, Little Chalfont, United Kingdom).

Electron Microscopy. For ultrastructural analysis, particles were transferred to Ni-grids coated with a Formvar film and stained with 1% aqueous uranyl acetate solution.

To detect protein A, the samples were fixed with 3% paraformaldehyde (Sigma), blocked with 1% acetylated BSA and 0.1% Tween 20 in PBS, and immunolabeled with a polyclonal GFP antibody (BD Biosciences Clontech, Mountain View, CA) and a secondary antibody conjugated with 10 nm gold (Sigma). After final washing with H₂O, the grids were stained with 1% uranyl acetate. The specimens were examined with a Zeiss EM 900 transmission electron microscope (Carl Zeiss SMT, Oberkochen, Germany).

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