(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
23 September 2010 (23.09.2010)

PCT

(10) International Publication Number
WO 2010/108182 A2

(51) International Patent Classification:
A61K 39/002 (2006.01)

(21) International Application Number:
PCT/US2010/028165

(22) International Filing Date:
22 March 2010 (22.03.2010)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

61/162,059	20 March 2009 (20.03.2009)	US
61/162,030	20 March 2009 (20.03.2009)	US
61/255,186	27 October 2009 (27.10.2009)	US

(71) Applicant *(for all designated States except US)*: **TETRAGENETICS, INC.**; One Broadway, 14th Floor, Cambridge, MA 02142 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **CLARK, Theodore, G.** [US/US]; 206 Fairmount Street, Ithaca, NY 14850 (US). **PAPOYAN, Ashot** [AM/US]; 8 Asbury Road, Lansing, NY 14882 (US). **TURKEWITZ, Aaron** [US/US]; 5410 S. Blackstone Avenue, Chicago, IL 60615 (US).

(74) Agents: **TWOMEY, Michael, J.** et al.; Wilmer Cutler Pickering Hale and Dorr LLP, 60 State Street, Boston, MA 02109 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2010/108182 A2

(54) Title: PRODUCTION OF RECOMBINANT PROTEINS IN CILIATES AND USES THEREOF

(57) Abstract: This invention is directed to methods for recombinant polypeptide production and, in particular, methods and products for the production and purification of recombinant proteins in ciliates.

PRODUCTION OF RECOMBINANT PROTEINS IN CILIATES AND USES THEREOF

[0001] This application claims priority to provisional U.S. Application Serial No. 61/162,059, filed on March 20, 2009, provisional U.S. Application Serial No. 61/162,030, filed on March 20, 2009 and provisional U.S. Application Serial No. 61/255,186, filed on October 27, 2009, which are each herein incorporated by reference in their entirety.

FIELD OF THE INVENTION

[0002] The invention relates to recombinant protein production and, in particular, methods and products for the production and purification of recombinant proteins in ciliates.

BACKGROUND OF THE INVENTION

[0003] Recombinant proteins are useful for a wide range of applications including, but not limited to, chemical and biological defense and the treatment and prevention of disease. Production of genetically engineered vaccine antigens, therapeutic proteins (including antibodies and antibody fragments), industrial enzymes, biopolymers, and bioremediation agents now constitute a multibillion dollar-per-year industry. There is also a large market for recombinant proteins in basic research (Pavlou and Reichert (2004); Langer (2005)).

[0004] Current platforms for the production of recombinant proteins are limited to a relatively small number of cell-based systems that include bacteria, fungi, and insect and mammalian tissue culture cells. Although bacteria can offer high yield and low cost alternatives for production of mammalian proteins, cell culture systems based on higher organisms (*e.g.*, insect cells or mammalian cell systems) generally provide proteins having greater fidelity to the natural proteins in terms of protein folding and/or post-translational processing (*e.g.*, glycosylation). Whole transgenic plants and animals have also been harnessed for the production of recombinant proteins, but the long development time from gene to final product can be a major drawback with these multicellular organisms, and purification of the recombinant proteins can be difficult and yield may be low.

[0005] Unicellular eukaryotes (*e.g.*, *Saccharomyces cerevisiae* and *Pichia pastoris*) grow rapidly in inexpensive media and share some common pathways of protein folding, post-translational modification and protein targeting with more advanced organisms

such as mammalian cells. Although the use of such unicellular eukaryotes for heterologous protein expression systems is known in the art, their rigid cell walls are an impediment to downstream protein purification.

[0006] After production of a desired recombinant protein within cells, the first step in isolating the protein is typically lysis of the cells. Lysis causes a forced mixing with the myriad of other cellular components, including proteases, which greatly complicates purification. In addition, lysis is problematic in expression systems that use microbial cells having rigid cell walls because the cell walls can impede downstream purification.

[0007] Although there are known methods, such as conventional chromatographic techniques (*e.g.*, ion-exchange and affinity chromatography), for separating a desired protein from a mixture of proteins and/or cellular debris, such techniques can be inefficient and can require successive rounds of isolation over expensive column matrices to obtain highly purified products. These drawbacks add to manufacturing costs. Purification of recombinant proteins is a key factor in production costs, and even the most efficient systems consume between 25% and 80% of capital costs in the purification process (Frankel (2000)).

[0008] Most eukaryotic cells are capable of constitutive secretion. This is a process whereby proteins are delivered to the extracellular space via cargo vesicles that traffic to the cell surface by way of the endoplasmic reticulum (ER) and Golgi (Burgess and Kelly (1987)). This pathway has been harnessed for the production of recombinant gene products in a variety of systems and has significant advantages for protein purification because the process of secretion separates proteins of interest from the bulk of contaminating cellular material and obviates the need for cell lysis. Nonetheless, constitutive secretion has drawbacks as well. Typically, the process is slow and requires days to weeks to generate sufficient yields of a recombinant polypeptide for commercial use. In addition, thermal denaturation and the presence of proteolytic enzymes released into the culture medium can adversely affect the uniformity and function of the final protein product.

[0009] While most cells (including eukaryotic microbes) secrete proteins constitutively, there are some specialized cells that also store proteins in cortical secretory organelles (granules), which they discharge in a stimulus-dependent or regulated fashion (Burgess and Kelly (1987); Miller and Moore (1990); Gundelfinger *et al.* (2003)). In contrast with constitutive secretion, regulated secretion is triggered by the presence of chemical mediators known as secretagogues. Such mediators cause increased levels of intracellular

calcium (Ca^{++}) which, in turn, trigger fusion of cortical granules with the plasma membrane and release of the granules contents into the surrounding extracellular space. Depending on the level of the stimulus, regulated secretion can be an all or none phenomenon. In some cases, relatively large amounts of protein can be released within a period on the order of milliseconds. The principal advantage of regulated secretion is that recombinant proteins can be harvested rapidly, thus speeding the manufacturing process, and improving the quality of the final product, particularly when long incubation times have deleterious effects on protein function.

[0010] Stimulus-dependent secretion has been intensively studied in specialized mammalian cells such as neurons, β-cells of the pancreas, and mast cells, and methods for the production of recombinant proteins that rely on regulated secretion have been described in the prior art (*e.g.*, U.S. Pat. No. 6,087,129; U.S. Pat. No. 6,110,707; U.S. Pat. No. 6,194,176; Grampp *et al.* (1992); Chen *et al.* (1995); Yang and Hsieh (2001)). These methods are drawn to the use of mammalian cells, and require that the gene for a protein that normally occupies the secretory granules (for example, insulin) be deleted and replaced by a gene for the recombinant protein (for example, prolactin) engineered to traffic to the same organelles. In all cases, the released proteins must be purified from culture supernatants using conventional chromatographic techniques following the addition of secretagogues to the growth media.

[0011] The use of mammal cells for the preparation of recombinant polypeptides can be further complicated by high costs and safety issues arising from the risks of mycoplasma or viral infections of the cell lines.

[0012] Therefore, there remains a need in the art for improved methods for rapid, high-fidelity and cost-effective production and purification of recombinant polypeptides.

SUMMARY OF THE INVENTION

[0013] In one aspect, the invention provides methods for producing a desired heterologous polypeptide in a culture of ciliates, by (a) expressing a fusion protein comprising the heterologous polypeptide and a polypeptide comprising at least one mucocyst-targeting polypeptide in the ciliates; (b) stimulating regulated secretion from mucocysts of the ciliates, whereby an extracellular matrix is formed by the secretion; (c) separating the extracellular matrix from the ciliates; and (d) isolating the fusion protein from the extracellular matrix (e.g. a recombinant soluble protein) in a ciliate by targeting the

heterologous polypeptide to mucocysts as a fusion protein with a mucocyst-targeting sequence or endogenous soluble mucocyst-targeted protein, stimulating regulated secretion from the mucocysts, and purifying the heterologous fusion protein from the resulting extracellular matrix produced by the mucocyst discharge. In each embodiment, the fusion protein comprises a polypeptide which targets the fusion protein to the mucocysts, and which includes a sequence which is cleaved by a protease endogenous to the mucocysts. As a result, all or some of the mucocyst-targeting sequences are removed from the heterologous polypeptide *in vivo*, enhancing its solubility, and facilitating its separation from the extracellular matrix produced by discharge of the mucocysts.

[0014] In another aspect, the invention provides isolated nucleic acids having a sequence encoding a fusion protein comprising: (a) at least one mucocyst-targeting polypeptide; (b) a heterologous polypeptide; and (c) a cleavable linker between the mucocyst-targeting polypeptide and the heterologous polypeptide.

[0015] In another aspect, the invention provides transgenic ciliates comprising: a nucleic acid having a sequence encoding: (a) at least one mucocyst-targeting polypeptide; (b) a heterologous polypeptide; and (c) a cleavable linker between the mucocyst-targeting polypeptide and the heterologous polypeptide.

[0016] In another aspect, the invention provides protein preparations comprising: (a) an extracellular matrix formed by regulated secretion by ciliates; and (b) a fusion protein encoded by the ciliates.

[0017] In another aspect, the invention provides vaccine preparations comprising: (a) an extracellular matrix formed by regulated secretion by ciliates; and (b) a fusion protein encoded by the ciliates; wherein the fusion protein comprises an immunogenic polypeptide.

[0018] In another aspect, the invention provides vaccine preparations comprising: (a) an extracellular matrix formed by regulated secretion by ciliates; and (b) at least two fusion proteins encoded by the ciliates; wherein the fusion proteins comprise different immunogenic polypeptides derived from the same pathogen or tumor cell.

[0019] In another aspect, the invention provides vaccine preparations comprising: (a) an extracellular matrix formed by regulated secretion by ciliates; and (b) at least two fusion proteins encoded by the ciliates; wherein the fusion proteins comprise different immunogenic polypeptides derived from different pathogens and/or tumor cells.

[0020] In another aspect, the invention provides vaccine preparations comprising: (a) an extracellular matrix formed by regulated secretion by ciliates; and (b) at least two different fusion proteins encoded by the ciliates; wherein one of the fusion proteins comprises an immunogenic polypeptide derived from a pathogen and/or tumor cell; and one of the fusion proteins comprises an immunostimulatory polypeptide or a receptor that binds an immunostimulatory polypeptide, designed to enhance the B- and/or T-cell response to the co-expressed immunogenic polypeptide(s).

[0021] Thus, in one aspect, the present invention provides methods for the production of a heterologous soluble polypeptide by a ciliate by (a) transforming the ciliate with a nucleic acid encoding a fusion protein including (i) a mucocyst-targeting polypeptide of a mucocyst-targeted protein which is cleaved by a protease endogenous to the mucocyst, and (ii) a heterologous polypeptide, such that expression of the fusion protein results in trafficking of the fusion protein to mucocysts within the ciliate and cleavage of the mucocyst-targeting polypeptide to release the heterologous soluble polypeptide within the mucocysts, (b) stimulating regulated secretion from the mucocysts of the ciliate, such that an extracellular matrix is formed, and (c) separating the heterologous soluble polypeptide from the extracellular matrix and the ciliates.

[0022] In some embodiments of this aspect, the mucocyst-targeted protein is a Grl protein, including a Grl-1 protein, such as a Grl-2 protein a Grl-3 protein, a Grl-4 protein, a Grl-5 protein, a Grl-6 protein, a Grl-7 protein, a Grl-8 protein, a Grl-9 protein or a Grl-10 protein, and in some embodiments the mucocyst-targeting domain comprises a pro-domain of a Grl protein. In other embodiments, the mucocyst-targeted protein is a β/γ crystalline domain containing protein.

[0023] In some embodiments of this aspect, the fusion protein also includes an endoplasmic reticulum-targeting polypeptide N-terminal to the mucocyst-targeting polypeptide. In some of these embodiments, the endoplasmic reticulum-targeting polypeptide is a pre-domain of a Grl protein, in some it is heterologous to said mucocyst-targeting polypeptide, and in some it is derived from an exogenous protein.

[0024] In another aspect, the invention provides methods for the production of a heterologous soluble polypeptide by a ciliate, by (a) transforming the ciliate with a nucleic acid encoding a first fusion protein including (i) a heterologous polypeptide, and (ii) at least a mucocyst-targeting polypeptide of a mucocyst-targeted protein, such that expression of the

first fusion protein results in trafficking of the first fusion protein to mucocysts within the ciliate, and such that an endogenous protease within the mucocysts cleaves a cleavage site within the mucocyst-targeting polypeptide and removes any sequences C-terminal to the cleavage site, thereby producing a second fusion protein within the mucocysts, (b) stimulating regulated secretion from the mucocysts of the ciliate, such that an extracellular matrix is formed, and (c) separating the heterologous polypeptide from the extracellular matrix and the ciliates.

[0025] In some embodiments of this aspect, the mucocyst-targeted protein is a Grl protein, including a Grl-1 protein, such as a Grl-2 protein a Grl-3 protein, a Grl-4 protein, a Grl-5 protein, a Grl-6 protein, a Grl-7 protein, a Grl-8 protein, a Grl-9 protein or a Grl-10 protein, and in some embodiments the mucocyst-targeting domain comprises a pro-domain of a Grl protein. In other embodiments, the mucocyst-targeted protein is a β/γ crystalline domain containing protein.

[0026] In some embodiments of this aspect, the fusion protein also includes an endoplasmic reticulum-targeting polypeptide N-terminal to the mucocyst-targeting polypeptide. In some of these embodiments, the endoplasmic reticulum-targeting polypeptide is a pre-domain of a Grl protein, in some it is heterologous to said mucocyst-targeting polypeptide, and in some it is derived from an exogenous protein.

[0027] In some embodiments of this aspect, the fusion protein also includes a second protease cleavage site between the heterologous polypeptide and the mucocyst-targeting polypeptide. In these embodiments, the method can also include the additional step of reacting said second fusion protein with a second protease which cleaves said second protease cleavage site after either step (b) or step (c).

[0028] In another aspect, the invention provides methods for the production of a heterologous soluble polypeptide by a ciliate by (a) transforming the ciliate with a nucleic acid encoding a fusion protein including (i) a soluble polypeptide endogenous to the mucocyst, (ii) a protease cleavage site, and (iii) a heterologous polypeptide, such that expression of the fusion protein results in trafficking of the fusion protein to mucocysts within the ciliate, (b) stimulating regulated secretion from the mucocysts of the ciliate, such that an extracellular matrix is formed by the secretion, (c) separating the fusion protein from the extracellular matrix and the ciliates, and (d) obtaining the heterologous soluble polypeptide from the fusion protein.

[0029] In some embodiments of this aspect, the mucocyst-targeted protein is an Igr protein. In some embodiments, the mucocyst-targeted protein is a granule tip protein. In other embodiments, the mucocyst-targeted protein is a β/γ crystalline domain containing protein. In other embodiments, the mucocyst-targeted protein is a C-terminal crystallin fold containing protein.

[0030] In some embodiments of this aspect, step (d) of the methods include reacting the heterologous soluble polypeptide with a protease which cleaves the protease cleavage site.

[0031] In some embodiments of this aspect, the fusion protein further comprises an endoplasmic reticulum-targeting polypeptide N-terminal to the heterologous polypeptide. In some of these embodiments, expression of the fusion protein results in trafficking of the fusion protein to mucocysts within the ciliate and cleavage of the endoplasmic reticulum-targeting polypeptide. In some of these embodiments, the endoplasmic reticulum-targeting polypeptide is a pre-domain of a Grl protein, in some embodiments it is heterologous to the endogenous soluble polypeptide, and in some embodiments it is exogenous to the ciliate.

[0032] In another aspect, the invention provides nucleic acids having a sequence encoding any of the fusion proteins described above. These nucleic acids can be isolated linear DNA molecules or can be integrated into various vectors for molecular cloning or transformation. The nucleic acids can also include regulatory regions such as promoters, terminators and enhancers to which the coding sequences are operably joined, as well as reporter an/or selectable marker genes.

[0033] In another aspect, the invention provides transgenic ciliates transformed with any of the nucleic acids of the invention.

[0034] In another aspect, the invention provides protein preparations produced by any of the methods of the invention. These protein preparations include crude preparations resulting from regulated secretion by the ciliates of the invention with minimal purification, as well as substantially pure preparations of the desired soluble heterologous polypeptides.

[0035] These and other aspects of the invention will be apparent to those of ordinary skill in the art in view of the following detailed description and examples.

BRIEF DESCRIPTION OF THE FIGURES

[0036] **Figure 1.**Immunofluorescence light micrograph of *T. thermophila.* The cell is stained with anti-tubulin antibodies to visualize the cilia (hair-like projections at the cell periphery) and DAPI to visualize the nucleus (round body at the lower center). The cell dimensions are ~ 20x50 μM.

[0037] **Figure 2.** Stimulus-dependent or regulated secretion in a ciliate, *Tetrahymena*. Top Left: *Tetrahymena* cell with surface-associated cilia. Bottom Left: Cross-section through the cell revealing large numbers of secretory granules (mucocysts) within the cortical cytoplasm. Top Center. Left-hand panel is a transmission electron micrograph showing a single granule docked at the plasma membrane. Right-hand panel is a confocal immunofluorescence image of granules aligned along ciliary rows containing an apically localized granule lattice protein (Bowman *et al.* (2005a)). Bottom Center. Following treatment of cells with secretagogues, the granules fuse with the plasma membrane and synchronously discharge their contents to the extracellular space. Once hydrated, the granule lattice proteins form an insoluble proteinaceous gel. Top Right. A cell culture induced to secrete was spun at 4,000 X g for 10 min. A white, packed cell pellet is visible at the bottom of the tube. The hydrated gel released from mucocysts is present just above the cell pellet and can be readily harvested with a spatula.

[0038] **Figure 3.** Fusion protein constructs for expression of soluble recombinant proteins in ciliates through the regulated secretion pathway. In Construct #1, N-terminally to C-terminally, a pre-domain and a pro-domain which result in trafficking to a mucocyst are fused to a heterologous polypeptide (HSP). When the fusion protein is trafficked to a mucocyst, the pre- and pro-domains are proteolytically removed, resulting in release of the heterologous polypeptide (HSP) within the mucocyst. In Construct #2, , N-terminally to C-terminally, a pre-domain is fused to the heterologous polypeptide (HSP), which is fused to a protease cleavage site (CS), which is fused to a pro-domain of a mucocyst-targeted protein, which is fused to at least a portion of a mature mucocyst-targeted protein. When the fusion protein is trafficked to a mucocyst, the pre- and pro-domains are proteolytically removed, resulting in release of the fusion of the heterologous polypeptide (HSP), cleavage site (CS) and pro-domain. After regulated secretion, this fusion can be treated with the corresponding protease to release the heterologous polypeptide (HSP), before or after separating it from the extracellular matrix formed by mucocyst discharge. In Construct #3, N-terminally to C-terminally, a pre-domain is fused to a soluble polypeptide (SP) endogenous to the mucocyst,

which is fused to a protease cleavage site (CS), which is fused to a heterologous polypeptide (HSP). When the fusion protein is trafficked to a mucocyst, the pre-domain is proteolytically removed, resulting in release of the fusion of the endogenous soluble polypeptide (SP), cleavage site (CS), and heterologous polypeptide (HSP). After regulated secretion, this fusion can be treated with the corresponding protease to release the heterologous polypeptide (HSP), before or after separating it from the extracellular matrix formed by mucocyst discharge.

[0039] **Figure 4.** Chimeric Grl1p:H5 and Grl1p:scFv gene products. Panel (A) shows a diagram of the fusion protein between the full-length, Grl1p, granule lattice protein from *T. thermophila* and the coding sequence of the influenza virus H5 hemagglutinin. The primary amino acid sequence of the protein is shown below with the H5 region shaded gray. The H5 protein lacks the signal peptide at its N-terminus as well as the single membrane-spanning domain at its C-terminus. Panel (B) shows a diagram of the fusion protein between Grl1p and a single-chain antibody Fv fragment against anthrax PA toxin. A 6x his and HA-epitope tag (YPYDVPDYA) was introduced immediately upstream of the first amino acid residue in the coding region for the scFv fragment and a TEV cleavage sequence (ENLYFQG) was placed between the N- and C-terminal fusion partners.

[0040] **Figure 5.** Trafficking of fusion proteins to secretory granules in *T. thermophila*. Sequences encoding the H5 hemagglutinin of the H5N1 strain of avian influenza virus (panels A, B) and a single-chain antibody fragment against anthrax PA toxin (tagged with HA, panels C, D) were linked C-terminally to the full-length coding sequence for Grl1p from *T. thermophila*. The resulting chimeric genes (*Grl1p:H5* and *Grl1p:scFab*) were introduced into the cadmium-responsive *MTT1* locus of *T. thermophila* and induced with 2 μg/ml $CdCl_2$. Cells were then fixed and permeabilized with detergent in order to localize the recombinant gene products by immunofluorescence microscopy. For H5 localization, permeabilized cells were incubated with a 1:50 dilution of the mouse mAb 5C5, which is specific for the H5 hemagglutinin, followed by a 1:200 dilution of rhodamine-tagged goat anti-mouse IgG. Panel (A) shows a stacked Z-series. Panel (B) shows a single Z-section through the cells. Note the obvious punctate staining at the cell periphery where cortical secretory granules are located. For localization of the scFab, cells were incubated in a 1:300 dilution of the mouse mAb against the HA epitope, followed by 1:500 dilution of rhodamine-tagged goat anti-mouse IgG. A similar pattern of staining was seen as with the

Grl1p:H5 fusions. Panel (C) shows confocal Z-section of a single cell, while panel (D) shows three cells at a slightly lower magnification.

[0041] Figure 6. Western blot of the recombinant *Grl1p:H5* protein secreted from *T. thermophila* in response to dibucaine. Cells transformed with the gene for the *GrlP1:H5* fusion protein were fixed, permeabilized and reacted sequentially with the mouse mAb 5C5 against the H5 hemagglutinin followed by goat anti-mouse IgG coupled to Texas Red. The panel at the left shows an immunofluorescence confocal image localizing the chimeric protein to cortical mucocysts. Live cells expressing the chimeric protein were harvested by low-speed centrifugation and the spent culture medium retained. Cells were then washed in buffer and induced to secrete their mucocyst contents by treatment with 20 mM dibucaine. After low speed centrifugation, the cell pellet, mucus layer and supernatant fractions (center panel) were separated, and equivalent volumes from each sample fractionated by SDS-PAGE under non-reducing conditions. Proteins were then transferred to a nitrocellulose filter and subjected to Western blotting with mAb 5C5 (right hand panel). Lanes 2 and 3 (right-hand panel) contain protein from the cell pellet and mucus layer, respectively, following the addition of dibucaine to washed cells. Lane 4 represents the spent culture medium from cells expressing the Grl:H5 fusion protein. Lane 5 represents the soluble supernatant fraction from non-dibucaine treated cells after the removal of cells by low-speed centrifugation. Lane 6 represents the soluble supernatant fraction of dibucaine-treated samples after removal of cells and mucus by low speed centrifugation. The only fraction containing detectable protein is the mucus itself (lane 3). Note that the size of the fusion protein on Western blots (~250 kDa) is appreciably larger than its predicted size (80 kDa).

[0042] Figure 7. Release of a recombinant polypeptide from the mucocyst gel matrix by treatment with a site-specific protease. As diagrammed in panel A (top), a cleavable linker (the TEV protease cleavage site) was engineered between Grl1p and the C-terminal single-chain antibody Fv fragment (scFv) shown in Figure 4B. Following regulated secretion stimulated by dibucaine, the mucocyst gel matrix would be expected to contain the recombinant polypeptide (as in Figure 6 with the chimeric Grl1p:H5 protein). However, treatment of the gel matrix with TEV might be expected to cleave the protein and release the C-terminal scFv fragment into the supernatant as a soluble protein (Panel A). Panel B shows a Western blot that tracks the fate of the recombinant scFv fragment in various fractions following stimulus-dependent secretion from cells using antibodies against an HA-epitope tag

engineered into the protein (see Figure 4). Lanes 1 and 2 contain proteins from cell pellet and high-speed supernatant fractions, respectively, of cell cultures treated with dibucaine to release mucus. Lane 3 contains protein in the mucus fraction obtained after stimulus-dependent secretion. The presence of two bands in lane 3 may be due to incomplete processing of the pro-domain of the Grl1:FscFv fusion protein which would result in two proteins that differ by approximately 18 kDa in size. Lanes 4-11 show the high-speed (soluble) supernatant (even lanes) and insoluble mucus proteins following treatment of mucus with TEV protease for 1 (lanes 4 and 5), 2 (lanes 6 and 7), 3 (lanes 8 and 9) and 5 (lanes 10 and 11) hours. Note the appearance of soluble scFv fragments in all TEV-treated samples. It is estimated that 30-40% of the chimeric scFv protein appears in the soluble phase under the conditions used in this experiment. The resulting His-tagged protein could be readily purified on a Ni-NTA resin following release from the mucocyst gel.

[0043] **Figure 8.** Mucocyst targeting of H5ΔTMD^{ProGrl1}. Shown in Figure 8A H5ΔTMD^{ProGrl1} construct design and a schematic representation of the gene product and the corresponding amino acid sequence. This construct consists of the H5N1 hemagglutinin protein sequence (plain text) including the N-terminal signal peptide (bold and underlined text) but lacking the carboxy-terminal transmembrane domain. Immediately carboxy-terminal of the H5N1 hemagglutinin sequence is a 10 X His tag (bold, italicized and underlined text) and a TEV protease site (plain underlined text). Immediately carboxy-terminal of the TEV protease site is the Grl1 sequence comprising the pro-domain (bold italicized text) and the mature sequence (bold text) but lacking the pre-domain. Figure 8B show trafficking of H5ΔTMD^{ProGrl1} to secretory granules in Tetrahymena. As described herein Tetrahymena cells harboring H5ΔTMD^{ProGrl1} expression constructs were induced with $CdCl_2$, fixed and localization of fusion protein determined by immunofluorescence (left panel). Right panel shows a merged image of light and dark field views. Figure 8C shows H5ΔTMD^{ProGrl1} fusion gene is expressed, targeted to Tetrahymena mucocysts and recovered in the mucus phase following regulated secretion. As described herein Tetrahymena strains harboring H5ΔTMD^{ProGrl1} expression constructs were induced with $CdCl_2$ and then treated with dibucaine to stimulate regulated exocytosis. Three phases (CL, cell lysate; SN, supernatant; M, mucus) were harvested and analyzed by anti-hemagglutinin Western analysis using the conformation specific 5C5 anti-hemagglutinin antibody. At left is an actual representation of the three phases isolated following centrifugation. H5ΔTMD^{ProGrl1} is predominantly associated with the harvested mucus fraction.

[0044] **Figure 9.** Mucocyst targeting of H5ΔTMD^{PrePro}. Figure 9A shows H5ΔTMD^{PrePro} construct design and a schematic representation of the gene product and the corresponding amino acid sequence. This construct contains the Grl1 Pre (bold underlined text) and Pro (bold italicized text) domains N-terminal of the mature H5N1 hemagglutinin protein sequence (plain text) that lacks the native amino-terminal signal peptide and carboxy-terminal transmembrane domain. The fusion construct additionally contains a 10 x His tag at the carboxy-terminus (bold, italicized underlined text). Figure 9B shows trafficking of H5ΔTMD^{PrePro} to secretory granules in Tetrahymena. As described herein Tetrahymena cells harboring H5ΔTMD^{PrePro} expression constructs were induced with $CdCl_2$, fixed and localization of fusion protein determined by immunofluorescence (left panel). Right panel shows a merged image of light and dark field views. Figure 9C shows the H5ΔTMD^{PrePro} fusion gene is expressed, targeted to Tetrahymena mucocysts and recovered in the mucus phase following regulated secretion. As described herein Tetrahymena strains harboring H5ΔTMD^{PrePro} expression constructs were induced with $CdCl_2$ and then treated with dibucaine to stimulate regulated exocytosis. Three phases (CL, cell lysate; SN, supernatant; M, mucus) were harvested and analyzed by anti-hemagglutinin Western analysis using the conformation specific 5C5 anti-hemagglutinin antibody. At left is an actual representation of the three phases isolated following centrifugation. H5ΔTMD^{PrePro} is predominantly associated with the harvested mucus fraction.

[0045] **Figure 10.** Mucocyst targeting of H5ΔTMD^{Igr1}. Figure 10A shows H5ΔTMD^{Igr1} construct design and a schematic representation of the gene product and the corresponding amino acid sequence. This construct contains the Igr1 protein (bold text) including the Igr1 signal peptide (Bold, underlined text) and the mature H5N1 hemagglutinin protein sequence (plain text) that lacks the native amino-terminal signal peptide and carboxy-terminal transmembrane domain separated by a TEV protease site (underlined text). The fusion construct additionally contains a 10 x His tag at the carboxy-terminus (italicized text). Figure 10B shows trafficking of H5ΔTMD^{Igr1} to secretory granules in Tetrahymena. As described herein Tetrahymena cells harboring H5ΔTMD^{Igr1} expression constructs were induced with $CdCl_2$, fixed and localization of fusion protein determined by immunofluorescence. Figure 10C shows the H5ΔTMD^{Igr1} fusion gene is expressed, targeted to Tetrahymena mucocysts and recovered in the mucus phase following regulated secretion. As described herein Tetrahymena strains harboring H5ΔTMD^{Igr1} expression constructs were

induced with $CdCl_2$ and then treated with dibucaine to stimulate regulated exocytosis. Three phases (CL, cell lysate; SN, supernatant; M, mucus) were harvested and analyzed by anti-hemagglutinin Western analysis using the conformation specific 5C5 anti-hemagglutinin antibody. At left is an actual representation of the three phases isolated following centrifugation. $H5\Delta TMD^{Igr1}$ is predominantly associated with the harvested mucus fraction.

[0046] **Figure 11.** Mucocyst targeting of $EPO^{ProGrl1}$. Figure 11A shows $EPO^{ProGrl1}$ construct design and a schematic representation of the gene product and the corresponding amino acid sequence. This construct consists of the feline EPO protein sequence (plain text) including the N-terminal H5N1 hemagglutinin signal peptide (bold and underlined text). Immediately carboxy-terminal of the EPO sequence is a 10 X His tag (bold, italicized and underlined text) and a TEV protease site (plain underlined text). Immediately carboxy-terminal of the TEV protease site is the Grl1 sequence comprising the pro-domain (bold italicized text) and the mature sequence (bold text) but lacking the pre-domain. Figure 11B shows the $EPO^{ProGrl1}$ fusion gene is expressed, targeted to Tetrahymena mucocysts and recovered in the mucus phase following regulated secretion. As described herein Tetrahymena strains harboring $EPO^{ProGrl1}$ expression constructs were induced with $CdCl_2$ and then treated with dibucaine to stimulate regulated exocytosis. Three phases (CL, cell lysate; SN, supernatant; M, mucus) were harvested and analyzed by anti-EPO Western analysis. At left is an actual representation of the three phases isolated following centrifugation. $EPO^{ProGrl1}$ is predominantly associated with the harvested mucus fraction.

[0047] **Figure 12.** Mucocyst targeting of $scFv^{Grl1}$. Figure 12A shows $scFv^{Grl1}$construct design and a schematic representation of the gene product and the corresponding amino acid sequence. This construct contains the Tetrahymena Grl1 protein (bold text) including the Grl1 signal peptide pre- (Bold, underlined text) and pro- (bold, italicized text) domains fused to the anti-anthrax PA toxin single chain antibody (plain text). Dividing the Grl1 and scFv sequences are a TEV protease site (underlined text), a 6 x His tag (underlined, bold and italicized text) and a HA epitope tag (bold, italicized text). Figure 12B show trafficking of $scFv^{Grl1}$ to secretory granules in Tetrahymena. As described herein Tetrahymena cells harboring $scFv^{Grl1}$ expression constructs were induced with $CdCl_2$, fixed and localization of fusion protein determined by immunofluorescence using an anti-HA primary antibody and a rhodamine-conjugated secondary antibody. Figure 12C shows the $scFv^{Grl1}$ fusion gene is expressed, targeted to Tetrahymena mucocysts and recovered in the mucus phase following regulated secretion. As described herein Tetrahymena strains

harboring $scFv^{Grl1}$ expression constructs were induced with $CdCl_2$ and then treated with dibucaine to stimulate regulated exocytosis. Three phases (CL, cell lysate; SN, supernatant; M, mucus) were harvested and analyzed by anti-HA Western analysis. At left is an actual representation of the three phases isolated following centrifugation. $scFv^{Grl1}$ is predominantly associated with the harvested mucus fraction. Highlighted in the mucus fraction are two forms of the fusion protein that corresponds to unprocessed Grl1 fusion (ProGrl1-scFv) and processed Grl1 fusion where the PrePro domain have been cleaved leaving the mature Grl1 protein fused to scFv (Grl1-scFv).

[0048] **Figure 13.** Mucocyst targeting of $pfs48/45^{Grl4}$. Figure 13A shows $pfs48/45^{Grl4}$ construct design and a schematic representation of the gene product and the corresponding amino acid sequence. This construct contains the Tetrahymena Grl4 protein (bold text) including the Grl4 signal peptide pre- (Bold, underlined text) and pro- (bold, italicized text) domains fused to the malarial antigen pfs48/45 (plain text). Carboxy-terminus of pfs48/45 is a 6 x His tag (underlined text), the carboxy--terminal domain of the immobilization antigen variant B protein of *Ichthyophthirius multifiliis* (italic text) and a HA epitope tag (Bold, italicized and underlined text). Figure 13B shows trafficking of $pfs48/45^{Grl4}$ to secretory granules in Tetrahymena. As described herein Tetrahymena cells harboring $pfs48/45^{Grl4}$ expression constructs were induced with $CdCl_2$, fixed and localization of fusion protein determined by immunofluorescence using an anti-HA primary antibody and a rhodamine-conjugated secondary antibody. Figure 13C shows the $pfs48/45^{Grl4}$ fusion gene is expressed, targeted to Tetrahymena mucocysts and recovered in the mucus phase following regulated secretion. As described herein Tetrahymena strains harboring $pfs48/45^{Grl4}$ expression constructs were induced with $CdCl_2$ and then treated with dibucaine to stimulate regulated exocytosis. Mucus (M) and Cell lysate (CL) fraction before and after induction of regulated secretion were harvested and analyzed by anti-HA Western analysis. $pfs48/45^{Grl4}$ is predominantly associated with the harvested mucus fraction. In the mucus fraction three forms of the fusion protein correspond to unprocessed Grl4 fusion (approximately 80 kDa), processed Grl4 fusion where the PrePro domain have been cleaved leaving the mature Grl4 protein fused to pfs48/45 (50 kDa) and a third species (160 kDa) that most likely represents a dimerized form of the unprocessed Grl4 fusion.

[0049] **Figure 14.** Mucocyst targeting of $pfs48/45^{PrePro}$. Figure 14A shows $pfs48/45^{PrePro}$ construct design and a schematic representation of the gene product and the corresponding amino acid sequence. This construct contains the Tetrahymena Grl1 protein

Pre- (underlined, bold text) and Pro- (bold text) domains fused to the malarial antigen pfs48/45 (plain text). Carboxy-terminus of pfs48/45 is a 6 x His tag (underlined text), the carboxy--terminal domain of the immobilization antigen variant B protein of *Ichthyophthirius multifiliis* (italic text) and a HA epitope tag (Bold, italicized and underlined text). Figure 14B shows the pfs48/45^{PrePro} fusion gene is expressed, targeted to Tetrahymena mucocysts and recovered in the mucus phase following regulated secretion. As described herein Tetrahymena strains harboring pfs48/45^{PrePro} expression constructs were induced with $CdCl_2$ and then treated with dibucaine to stimulate regulated exocytosis. Mucus (M) and Cell lysate (CL) fraction before and after induction of regulated secretion were harvested and analyzed by anti-HA Western analysis. pfs48/45^{PrePro} is predominantly associated with the harvested mucus fraction.

[0050] Figure 15. Extraction and purification of H5ΔTMD^{ProGrl1}. Figure 15A shows extraction H5ΔTMD^{ProGrl1}. Cells were induced to express H5ΔTMD^{ProGrl1}, mucocyst contents released and soluble protein extracted from harvested mucus as described herein. Western analysis was carried out with the anti-H5N1 hemagglutinin antibody 5C5 as described above. Shown is the mucus fraction before and after extraction and the resulting soluble fraction. Highlighted are the H5ΔTMD-Grl1 fusion and H5ΔTMD monomer proteins. Figure 15B shows a Western analysis using the 5C5 antibody of the extracted soluble fraction with and without TEV protease treatment. Note the conversion of high-molecular weight H5ΔTMD^{ProGrl1} fusion protein to the H5ΔTMD monomer (~64 kDA). Figure 15C shows purification of H5ΔTMD by Ni-NTA affinity chromatography. TEV treated soluble extracted fraction was passed over Ni-NTA resin and eluted in buffer containing imidazole. Shown is a Western analysis using the 5C5 antibody of purification fractions. SN, Soluble extract; FT, Ni-NTA flow-through; W, wash fraction. Figure 15D shows SDS-PAGE analysis of purified soluble H5ΔTMD.

[0051] Figure 16. Extraction and purification of scFv^{Grl1}. Figure 16A shows extraction of scFv^{Grl1}. Mucus containing scFv^{Grl1} was treated directly with TEV protease as described herein. Anti-HA Western analysis was performed on fractionated samples: CL, Cell Lysate, SN, supernatant following regulated secretion; M, mucus; S, soluble fraction following treatment of Mucus with TEV protease. Highlighted are differentially processed forms of scFv^{Grl1} including soluble scFv monomer, Grl1-scFv where the Grl1 prodomain has been cleaved *in vivo*, and scFv^{Grl1} fusion protein (depicted as ProGrl1-scFv in this figure). Figure 16B shows purification of scFv by Ni-NTA affinity chromatography. Shown is an

anti-HA Western analysis of elution fractions following Ni-NTA chromatography of the soluble fraction derived from TEV treatment of mucus containing $scFv^{Grl1}$.

[0052] **Figure 17**. $H5\Delta TMD^{PrePro}$ and $H5\Delta TMD^{ProGrl1}$ fusion genes are expressed, targeted to Tetrahymena mucocysts and recovered in the mucus phase following regulated secretion. As described herein Tetrahymena strains harboring $H5\Delta TMD^{PrePro}$ and $H5\Delta TMD^{ProGrl1}$ expression constructs were induced with $CdCl_2$ and then treated with dibucaine to stimulate regulated exocytosis. Three phases (CL, cell lysate; SN, supernatant; M, mucus) were harvested and analyzed by anti-hemagglutinin Western analysis using the conformation specific 5C5 anti-hemagglutinin antibody. At left is an actual representation of the three phases isolated following centrifugation. Both $H5\Delta TMD^{PrePro}$ and $H5\Delta TMD^{ProGrl1}$ were predominantly associated with the harvested mucus fraction.

[0053] **Figure 18**. Mucus associated $H5\Delta TMD^{PrePro}$ and $H5\Delta TMD^{ProGrl1}$ elicit an immune response in animals. Rats were immunized with either mucus-associated $H5\Delta TMD^{PrePro}$ or mucus-associated $H5\Delta TMD^{ProGrl1}$. Three weeks post-injection sample bleeds were tested for the presence of anti-hemagglutinin antibody by Western analysis using a commercially available insect cell-derived recombinant H5N1 hemagglutinin. Both rat sera samples are positive for anti-hemagglutinin antibody (2 panels on left). Control westerns using either pre-immune sera or secondary antibody alone were negative (2 panels on right).

DETAILED DESCRIPTION OF THE INVENTION

[0054] All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. The patent, scientific and technical literature referred to herein establish knowledge that was available to those skilled in the art at the time of filing. The entire disclosures of the issued U.S. patents, published and pending patent applications, and other publications that are cited herein are hereby incorporated by reference to the same extent as if each was specifically and individually indicated to be incorporated by reference. In the case of any inconsistencies, the present disclosure will prevail.

[0055] Definitions.

[0056] All scientific and technical terms used herein, unless otherwise defined below, are intended to have the same meaning as commonly understood by one of ordinary

skill in the art. References to techniques employed herein are intended to refer to the techniques as commonly understood in the art, including variations on those techniques or substitutions of equivalent or later-developed techniques which would be apparent to one of skill in the art. In addition, in order to more clearly and concisely describe the subject matter which is the invention, the following definitions are provided for certain terms which are used in the specification and appended claims.

[0057] As used herein, the term "ciliates" means eukaryotes belonging to the kingdom Chromalveolata, the superphylum Alveolata, and the phylum Ciliophora. Ciliates are complex protozoa characterized by the presence of cilia on their cell surfaces and dimorphic nuclei consisting of a macronucleus and one or more micronuclei.

[0058] As used herein, "*Tetrahymena spp.*" refers to ciliate protozoa in the family of Tetrahymenidae. Exemplary *Tetrahymena spp.* include, but are not limited to, *T. thermophila* and *T. pyriformis*.

[0059] As used herein, the term the term "dense core granule" refers to a subset of the secretory organelles in ciliates that have electron dense cores and discharge in a stimulus-dependent fashion. Exemplary dense core granules include, but are not limited to, mucocysts in *Tetrahymena spp.* and trichocysts in *Paramecium spp.*

[0060] As used herein, the term "mucocyst" refers to secretory organelles in ciliates, also referred to as "cortical granules," that secrete or discharge a proteinaceous mucus in response to a secretory stimulus.

[0061] As used herein, a "secretory stimulus" refers to a condition or treatment that directly or indirectly stimulates or increases the release of a protein from a dense core granule (*e.g.*, a mucocyst). Exemplary secretory stimuli suitable for use with the methods disclosed herein include, but are no limited to, treatment with a secretagogue, mechanical shock, cross-linking of surface antigens and electroshock (*e.g.*, electroporation).

[0062] As used herein, the term "secretagogue" refers to a compound or agent that directly or indirectly stimulates or increases the release of a protein from a dense core granule (*e.g.*, a mucocyst). Exemplary secretagogues suitable for use with the methods disclosed herein include, but are no limited to, dibucaine, NaCl, Alcian blue, ~0.25M sucrose and compounds that increase intracellular Ca^{2+} levels (*e.g.*, calcium ionophores such as A23187).

[0063] The term "targeting polypeptide" means a polypeptide (a "secretory peptide") that, as a component of a larger polypeptide, directs the larger polypeptide through

a secretory pathway of a cell in which it is synthesized. The larger polypeptide can be cleaved to remove the secretory peptide during transit through the secretory pathway.

[0064] As used herein, the term "endoplasmic reticulum-targeting polypeptide" means a sequence of amino acids, present at the N-terminus of a polypeptide, that causes the polypeptide to be inserted into the endoplasmic reticulum (ER) upon synthesis. Endoplasmic reticulum-targeting polypeptides typically comprise 5-10 hydrophobic amino acids that bind to a signal recognition particle (SRP) which facilitates transport into the ER. Some endoplasmic reticulum-targeting polypeptides are cleaved from the polypeptide by a signal peptidase present within the ER. Endoplasmic reticulum-targeting polypeptides are a subset of the class of polypeptides variously known as leader sequences, signal sequences, targeting signals, transit peptides, or localization signals, which target polypeptides to organelles such as the nucleus, mitochondria, chloroplasts, secretory granules and ER. For some proteins, including ciliate Grl proteins, the endoplasmic reticulum-targeting polypeptide may be referred to as a "pre-domain."

[0065] As used herein, the term "mucocyst-targeting polypeptide" means a sequence of amino acids that causes the polypeptide to be trafficked into the cortical secretory granules (*i.e.*, mucocysts) of ciliates as the granules are formed. Mucocyst-targeting polypeptides typically are located at the N-terminus of the polypeptide, or immediately C-terminal to an endoplasmic reticulum-targeting polypeptide. Some mucocyst-targeting polypeptides are cleaved from the polypeptide by a site-specific protease present within the granules. Endoplasmic reticulum targeting polypeptides are a subset of the class of polypeptides variously known as leader sequences, signal sequences, targeting signals, transit peptides, or localization signals, which target polypeptides to organelles such as the nucleus, mitochondria, chloroplasts, secretory granules and ER. For some proteins, including ciliate Grl proteins, the mucocyst-targeting polypeptide may be referred to as a "pro-domain."

[0066] As used herein, the term "cleavage site" refers to a specific sequence of amino acids that can be cleaved specifically by a cleavage agent, such as a protease, or that self-cleaves, such as an intein sequence.

[0067] As used herein, the term "cleavable linker" refers to a sequence of amino acids that comprises a cleavage site and that joins two structural domains of a protein.

[0068] As used herein, the term "antibody" is intended to embrace naturally produced antibodies, recombinantly produced antibodies, monoclonal antibodies, and

polyclonal antibodies, as well as antibody fragments such as Fab fragments, $F(ab')_2$ fragments, Fv fragments, and single-chain Fv fragment (scFv). Useful antibodies include all immunoglobulin classes, such as IgM, IgG, IgD, IgE, IgA and their subclasses. Antibodies may be produced by standard methods, well known in the art. See, *e.g.,* Pluckthun (1990), *Nature* 347:497-498; Huse *et al.* (1989), *Science* 246:1275-1289; Chaudhary *et al.* (1990), *Proc. Natl. Acad. Sci. USA* 87:1066-1070; Mullinax *et al.* (1990), *Proc. Natl. Acad. Sci. USA* 87:8095-8099; Berg *et al.* (1991), *Proc. Natl. Acad. Sci. USA* 88:4723-4727; Wood *et al.* (1990), *J. Immunol.* 145:3011-3016; and references cited therein.

[0069] As used herein, the term "heterologous" means, with respect to two or more genetic or protein sequences, that the sequences do not occur in the same physical relation to each other in nature and/or do not naturally occur within the same genome or protein. For example, a genetic construct may include a coding sequence which is operably joined to one or more regulatory sequences, or to one or more other coding sequences, and these sequences are considered heterologous to each other if they are not operably joined in nature and/or they are not found in the same relation in a genome in nature. Similarly, a protein may include a first polypeptide sequence which is joined by a standard peptide bond to a second polypeptide sequence, and these sequences are considered heterologous to each other if they are not found in the same relation in any protein or proteome in nature.

[0070] As used herein, the term "endogenous" means, with respect to a genetic or protein sequence, that the sequence occurs naturally in the same physical relation to a specified sequence, or occurs naturally in a specified cell or genome. For example, a genetic construct may include a coding sequence which is operably joined to one or more regulatory sequences, and the regulatory sequences are considered endogenous if they are operably joined to the coding sequence in nature, and/or they are found in the same relation in a genome in nature. Similarly, a protein that occurs naturally in a specified cell type or species, is considered endogenous to that cell or species.

[0071] As used herein, the term "homolog" means a protein which is evolutionarily-related to and shares substantial structural and functional similarity with a reference protein in a different species (*e.g.*, *Tetrahymena spp.* Grl proteins).

[0072] As used herein, the term "promoter" means a nucleotide sequence which is capable of binding RNA polymerase and initiating transcription of a downstream or 3' coding sequence.

[0073] As used herein, the term "selectable marker" means any genetic sequence which, when expressed, has a biochemical or phenotypic effect which is dominant and selectable by the presence or absence of a selection agent.

[0074] As used herein with respect to protein preparations, the term "substantially pure" means a preparation which contains at least 60% (by dry weight) the protein of interest, exclusive of the weight of other intentionally included compounds. In some embodiments, the -preparation is at least 75%, at least 90%, or at least 99%, by dry weight the protein of interest, exclusive of the weight of other intentionally included compounds. Purity can be measured by any appropriate method, *e.g.*, column chromatography, gel electrophoresis, or HPLC analysis. If a preparation intentionally includes two or more different proteins of the invention, a "substantially pure" preparation means a preparation in which the total dry weight of the proteins of the invention is at least 60% of the total dry weight, exclusive of the weight of other intentionally included compounds. For such preparations containing two or more proteins of the invention, the total weight of the proteins of the invention can be at least 75%, at least 90%, or at least 99%, of the total dry weight of the preparation, exclusive of the weight of other intentionally included compounds. Thus, if the proteins of the invention are mixed with one or more other proteins(*e.g.*, serum albumin) or compounds (*e.g.*, diluents, detergents, excipients, salts, polysaccharides, sugars, lipids) for purposes of administration, stability, storage, and the like, the weight of such other proteins or compounds is ignored in the calculation of the purity of the preparation.

[0075] As used herein, the term "transform" means to introduce into a cell an exogenous nucleic acid or nucleic acid analog which replicates within that cell, that encodes a polypeptide sequence which is expressed in that cell (with or without integration into the genome of the cell), and/or that is integrated into the genome of that cell so as to affect the expression of a genetic locus within the genome. The term "transform" is used to embrace all of the various methods of introducing such nucleic acids or nucleic acid analogs, including, but not limited to the methods referred to in the art as transformation, transfection, transduction, or gene transfer, and including techniques such as microinjection, DEAE-dextran-mediated endocytosis, calcium phosphate coprecipitation, electroporation, liposome-mediated transfection, ballistic injection, viral-mediated transfection, and the like.

[0076] As used herein, the term "vector" means any genetic construct, such as a plasmid, phage, transposon, cosmid, chromosome, virus, virion, etc., which is capable transferring nucleic acids between cells. Vectors may be capable of one or more of

replication, expression, and insertion or integration, but need not possess each of these capabilities. Thus, the term includes cloning, expression, homologous recombination, and knock-out vectors.

[0077] As used herein, the terms "increase" and "decrease" mean, respectively, to cause an increase or decrease of at least 5%, as determined by a method and sample size that achieves statistically significance (*i.e.*, $p < 0.1$).

[0078] As used herein, the term "statistically significant" means having a probability of less than 10% under the relevant null hypothesis (*i.e.*, $p < 0.1$).

[0079] As used herein, the recitation of a numerical range for a variable is intended to convey that the invention may be practiced with the variable equal to any of the values within that range. Thus, for a variable that is inherently discrete, the variable can be equal to any integer value within the numerical range, including the end-points of the range. Similarly, for a variable that is inherently continuous, the variable can be equal to any real value within the numerical range, including the end-points of the range. As an example, and without limitation, a variable which is described as having values between 0 and 2 can take the values 0, 1 or 2 if the variable is inherently discrete, and can take the values 0.0, 0.1, 0.01, 0.001, . . . , 0.9, 0.99, 0.999, or any other real values ≥ 0 and ≤ 2, if the variable is inherently continuous.

[0080] As used herein, unless specifically indicated otherwise, the word "or" is used in the inclusive sense of "and/or" and not the exclusive sense of "either/or."

[0081] As used herein and in the appended claims, the use of singular forms of words, and the use of the singular articles "a," "an" and "the," are intended to include and not exclude the use of a plurality of the referenced term unless the content clearly dictates otherwise.

[0082] The present invention provides methods and compositions for producing a desired heterologous polypeptide in a ciliate (*e.g.*, *Tetrahymena thermophila* or *Tetrahymena pyriformis)* by targeting the heterologous polypeptide to mucocysts as a fusion protein with a mucocyst-targeting sequence or endogenous soluble mucocyst-targeted protein, stimulating regulated secretion from the mucocysts, and purifying the heterologous fusion protein from the resulting extra-cellular matrix produced by the mucocyst discharge.

[0083] In some embodiments, the fusion protein comprises a polypeptide which targets the fusion protein to the mucocysts, and which includes a sequence which is cleaved by a protease endogenous to the mucocysts. As a result, all or some of the mucocyst-targeting sequences are removed from the heterologous polypeptide *in vivo*, enhancing its solubility, and facilitating its separation from the extracellular matrix produced by discharge of the mucocysts.

[0084] The proteins stored by ciliates are distinctive in terms of their structures and ability to self-associate upon granule discharge. Whereas the proteins released naturally by mammalian cells are soluble following exocytosis, the majority of proteins discharged from storage granules of ciliates self-associate, forming large macromolecular aggregates. In certain embodiments, the soluble recombinant proteins of the invention, can be separated from the insoluble endogenous mucocyst proteins which form an extracellular matrix after regulated secretion.

[0085] Thus, in one aspect the invention employs a different approach to recombinant protein production than in prior art methods which require lysis of the cells followed by purification from the lysate, or which require constitutive expression or regulated secretion of soluble proteins into a culture medium followed by purification from the medium. In certain embodiments, the invention exploits the regulated and simultaneous discharge of mucocysts to secrete the desired soluble recombinant proteins at high concentration (rather than the low concentration that usually results from slow, continuous secretion into culture medium), and further exploits the insoluble nature of the extracellular matrix material to separate the endogenous mucocyst proteins from the desired soluble recombinant proteins.

[0086] In certain embodiments, the invention relies on the natural, insoluble matrix material secreted by ciliates in order to obtain highly purified recombinant proteins in a simple, one-to-two step process. To accomplish this, molecular cloning techniques are used to direct fusion proteins comprising desired heterologous polypeptides to the cortical mucocysts of the ciliates by linking them to one or more mucocyst-targeting polypeptides. In certain embodiments, the mucocyst-targeting sequences are then cleaved by endogenous processes to release the desired soluble recombinant protein within the mucocysts. Mucocyst discharge is triggered with an appropriate stimulus to release the fusion proteins into the extracellular space in association with the proteinaceous mucocyst matrix.

[0087] In certain embodiments, the gel matrix is then harvested by low-speed centrifugation or filtration, and the desired a heterologous polypeptide is recovered in a purified form by dissociation (with or without cleavage from other fusion protein sequences) from the matrix. This approach permits purification of proteins to near homogeneity in a very rapid process that obviates the need for serial rounds of purification following cell lysis or secretion by conventional routes. In other embodiments, the gel matrix and intact ciliate cells are then harvested by low-speed centrifugation or filtration, and the desired heterologous polypeptide is recovered in a purified form by dissociation (with or without cleavage from other fusion protein sequences) from the matrix. This approach permits purification of proteins to near homogeneity in a very rapid process that obviates the need for serial rounds of purification following cell lysis or secretion by conventional routes.

[0088] Significantly, the present invention exploits (a) the limited number of proteins present in the mucocysts of ciliates to reduce the complexity of the protein mixture to be purified, (b) the regulated secretion mechanism of mucocysts to cause synchronized and nearly instantaneous secretion by a population of cells, and (c) the insoluble extracellular matrices produced by the mucocyst discharge for protein isolation.

[0089] In addition, as described below, the invention provides nucleic acid constructs encoding the fusion proteins of the invention, cassettes for producing such fusion proteins between targeting sequences and sequences encoding a desired heterologous polypeptide, methods for targeting a desired heterologous polypeptide to a mucocyst, methods for producing a desired heterologous polypeptide in a ciliate, methods for inducing the regulated secretion of a desired heterologous polypeptide from a ciliate, and methods for purifying a desired heterologous polypeptide from the extracellular matrix resulting from regulated secretion by the mucocysts.

[0090] Along with streamlined purification, a further advantage of this approach is the apparent absence of secreted proteases following regulated secretion from mucocysts. Whereas stimulus-dependent secretion in mammalian cells is typically accompanied by the release of lysosomal proteases that are potentially damaging to expressed recombinant polypeptides (Andrews (2000)), mass spectrophotometric analysis of proteins released from *Tetrahymena spp.* following regulated exocytosis has revealed a paucity of such enzymes, thus giving the ciliate expression system an advantage relative to mammalian expression systems. Therefore, because the methods described herein result in little or no release of lysosomal proteases into the medium upon regulated secretion, the invention provides for

improved protein preparations, with reduced levels of proteases and reduced levels of proteolytic fragments. Consequently, yield and fidelity are improved and production costs are reduced.

[0091] Mucocyst-Targeting of Heterologous Polypeptides

[0092] Ciliates engage in regulated secretion of proteins stored in cortical secretory organelles (granules), which are discharged in a stimulus-dependent or regulated fashion (Turkewitz *et al.* (2000); Turkewitz (2004)). In *Tetrahymena spp.*, these dense core granules are termed mucocysts.

[0093] Each *Tetrahymena spp.* cell contains numerous mucocysts docked at the plasma membrane. Upon stimulation, the discharge of the mucocyst contents occurs in a rapid and synchronous manner (Satir (1977)). The signal sequences that target proteins to the dense core granules are not yet well-characterized, but small stable loops appear to be important determinants in several systems (Chanat *et al.* (1993); Cool *et al.* (1995); Cool *et al.* (1997); Glombik *et al.* (1999); Roy *et al.* (1991); Zhang *et al.* (1999)), and the sequences are readily identified by deletion analysis. Regions of limited sequence similarity border known proteolytic processing sites in Grl proteins and accordingly may be targets for protease processing (Bradshaw *et al.* (2003)).

[0094] At least twelve proteins localize to mucocysts in *Tetrahymena spp.* (Chilcoat *et al.* (1996); Haddad *et al.* (2002); Bradshaw *et al.* (2003); Cowan *et al.* (2005); Bowman *et al.* (2005a)). The most abundant of these, known as granule lattice proteins (Grls), form a crystalline array that fills the granule space. The genome of *Tetrahymena spp.* contains at least ten GRL genes, and the granule cores in *Tetrahymena spp.* comprise a cargo of polypeptide-based lattices of proteins derived from proteolytically processed Grl precursors (Collins and Wilhelm (1981); Bradshaw *et al.* (2003)).

[0095] The invention employs fusion proteins of mucocyst-targeting polypeptides to direct the trafficking of a desired heterologous polypeptide to the mucocysts of a ciliate. In nature, polypeptides are trafficked to and between the membrane-bound compartments (*e.g.*, the endoplasmic reticulum, the Golgi apparatus, lysosomes, vacuoles, secretory vesicles or granules, etc.) based, in part, upon the presence of N-terminal "leader sequences" or "signal sequences." These same targeting sequences can be employed to target heterologous proteins to desired compartments.

[0096] Ciliates, such as *Tetrahymena*, also have a constitutive secretory pathway through which many secretory proteins are released. However, the constitutive secretory route does not contribute to the release of Grl proteins, indicating that sorting between the pathways of regulatory secretion and constitutive secretion occurs in *Tetrahymena spp.*

[0097] For targeting polypeptides to the mucocysts of ciliates, any of the naturally-occurring targeting sequences of naturally-occurring granule lattice mucocyst proteins can be employed. For example, the signal sequences for Grlp1 have been identified (Chilcoat *et al.* (1996)) and can be used to direct a fusion protein comprising the signal sequences to the cortical secretory granules in *Tetrahymena spp.* In addition to the N-terminal leader or signal sequences, larger fragments of endogenous mucocyst proteins can be fused to the desired heterologous polypeptides, as long as these larger fragments can include the targeting sequences necessary for trafficking the fusion protein to the mucocysts. For example, entire N-terminal structural domains, or an entire mucocyst-targeted protein, can be fused to the heterologous polypeptide and used as a targeting sequence.

[0098] *Tetrahymena thermophila* Grl sequences include, but are not limited to, the Granule Lattice Protein 1 Precursor (SEQ ID NO: 1), Granule Lattice Protein 3 Precursor (SEQ ID NO: 2), Granule Lattice Protein 4 Precursor (SEQ ID NO: 3), Granule Lattice Protein 5 Precursor (SEQ ID NO: 4), and Granule Lattice Protein 7 Precursor (SEQ ID NO: 5). The sequences of homologs from other *Tetrahymena* and other ciliate species are known in the art or can be determined, and these homologs can be used in the inventions described herein.

[0099] Granule lattice protein 2 precursor, granule lattice protein 6 precursor, granule lattice protein 9 precursor and granule lattice protein 10 precursor are also suitable for use as targeting sequences in conjunction with the methods and compositions disclosed herein.

[00100] Genetic Constructs for Fusion Proteins.

[00101] As described herein, a desired heterologous polypeptide can be produced as a fusion protein with a mucocyst-targeting polypeptide. The targeting polypeptide can be an N-terminal leader or signal sequence from an endogenous mucocyst protein, can be a larger fragment of the mucocyst protein, or can be the entire mucocyst protein or a functional homolog thereof.

[00102] In accordance with the invention, the mucocyst-targeting polypeptide can comprise a Grl polypeptide, a Grl pre-protein polypeptide, a truncation product of a Grl protein, a fragment of a Grl polypeptide, a polypeptide that is homologous to a Grl, a polypeptide or a polypeptide having a sequence at least 70% identical to the amino acid sequence of a Grl protein and exhibiting mucocyst-targeting activity. In some embodiments, the mucocyst-targeting polypeptide has at least 99%, 97%, 95%, 90%, 80% or 70% amino acid sequence identity to the amino acid sequence of a Grl protein.

[00103] Genetic constructs encoding such fusion proteins can readily be prepared by one of skill in the art based upon the universal genetic code, and optionally employing the codon preferences characteristic of the ciliate host. See Larsen *et al.* (1999); Wuitschick and Karrer (2000); Eisen *et al.* (2006); and Wuitschick and Karrer (1999).

[00104] The genetic constructs can be designed to include a cleavable linker such as protease cleavage site, self-cleaving intein sequence, or flexible linker sequence between the mucocyst targeting polypeptide(s) and the heterologous polypeptide, and/or may be designed to include additional sequences useful for purification of the fusion protein (*e.g.*, poly-His or epitope tags for affinity or immuno purification).

[00105] The sequences encoding the fusion protein can be introduced into the cells on expression plasmids, or can be stably integrated into the protist genome (*e.g.*, by homologous recombination, retroviral insertion). When integrated into the genome, the fusion protein sequences can replace (in whole or in part) the endogenous sequences encoding the corresponding mucocyst protein, or can be inserted at a separate genomic location. Targeting sequences useful for secretion of foreign proteins in *Tetrahymena spp.* are described in (Clark *et al.* (2001)).

[00106] The nucleic acid sequences can be cloned using standard cloning procedures in the art, as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Springs Laboratory, Cold Springs Harbor, N.Y. (1989). For example, chimeric genes encoding the fusion proteins can be generated by linking coding regions of genes for the heterologous polypeptides to endogenous mucocyst targeting polypeptides (or mucocyst protein fragments or entire mucocyst proteins) either synthetically (38), or by PCR using serial overlap extension. The resulting constructs can then introduced into standard plasmid DNA vectors (*e.g.*, TOPO, BlueScript, etc.) for amplification in *E. coli* by chemical transformation, electroporation or any other method known in the art.

[00107] Inducing Regulated Secretion in Ciliates.

[00108] Mucocyst discharge can be triggered with appropriate secretory stimuli to release mucocyst-targeted heterologous proteins into the extracellular space in association with the proteinaceous mucocyst gel. Regulated secretion can depend on the level of the stimulus, and can be an all-or-none phenomenon with, in some cases, large amounts of protein being released within a short period of time (on the order of milliseconds). For example, treatment of *Tetrahymena spp.* cells with dibucaine, or other secretagogues, results in rapid fusion of mucocyst membranes with the plasma membrane, and discharge of the granule contents into the extracellular space (Turkewitz *et al.* (2000); Turkewitz (2004); Maihle and Satir (1986)) (Figure 2). A single *Tetrahymena spp.* cell can store large amounts of protein in its roughly 4,500 mucocysts (Turkewitz *et al.* (2000); Turkewitz (2004); Chilcoat *et al.* (1996); Haddad *et al.* (2002); Cowan *et al.* (2005); Bowman *et al.* (2005a); Bradshaw *et al.* (2003); Bowman *et al.* (2005b)).

[00109] Regulated secretion can be triggered by the presence of chemical mediators known as secretagogues. For example, such mediators can cause increased levels of intracellular calcium (Ca^{2+}), which, in turn, trigger fusion of cortical granules with the plasma membrane resulting in a release of the granule contents into the surrounding extracellular space. Examples of secretagogues useful in the invention include, but are not limited to, dibucaine, Alcian blue, elevated NaCl, sucrose and Ca^{2+} ionophores.

[00110] Regulated secretion can also be triggered by secretory stimuli other than secretagogues. Examples of such secretory stimuli useful in the invention include, but are not limited to, treatment with mechanical shock, cross-linking of surface antigens, and electroshock (*e.g.*, electroporation).

[00111] Unlike regulated secretion in mammalian systems, some proteins stored by the mucocysts of ciliates do not remain soluble following exocytosis. Proteins discharged from ciliated protozoa such as *Tetrahymena* and *Paramecium* self-associate upon granule discharge and form macromolecular aggregates. In the case of *Paramecium*, the released material forms trichocysts (spear-like projections) that protrude from the cell on granule discharge (Madeddu *et al.* (1994); Vayssié *et al.* (2000)). In *Tetrahymena spp.*, the granule contents take the form of a proteinaceous gel when shed (*e.g.*, similar in consistency to agarose or Sepharose). This gel can surround the cell in a transparent, mucus-like capsule (Turkewitz *et al.* (2000); Turkewitz (2004)). In *Ichthyophthirius multifiliis*, i-antigen

clustering causes the formation of a similar gel via triggered secretion of cortical mucocysts. Regulated secretion is also known to occur in *Paramecium tetraurelia*.

[00112] Genetic Constructs for Fusion Proteins.

[00113] As described herein, a desired heterologous polypeptide can be produced as a fusion protein with one or more mucocyst-targeting sequences. The targeting sequence can be an N-terminal leader or signal sequence from an endogenous mucocyst protein, can be a larger fragment of the mucocyst protein, or can be the entire mucocyst protein or a functional homolog thereof. However, within the mucocysts, the endogenous processing machinery of the granules can be used to cleave away the mucocyst-targeting sequences and thereby generate recombinant proteins that are freely soluble within cortical mucocysts *in vivo*. After inducing regulated secretion by the mucocysts, the heterologous soluble polypeptide can be isolated from the resulting insoluble extracellular matrix.

[00114] The invention provides three distinct forms of genetic construct for achieving this objective. Exemplary constructs are illustrated schematically in Figure 3, where the desired heterologous soluble polypeptide is indicated as "HSP," the endoplasmic reticulum-targeting polypeptide is indicated as "pre," the mucocyst-targeting polypeptide is indicated as "pro," an endogenous soluble mucocyst-targeted protein is indicated as "SP," and a protease cleavage site is indicated as "CS."

[00115] In a first series of embodiments, the fusion protein comprises, N-terminally to C-terminally, (a) a mucocyst-targeting polypeptide of a mucocyst-targeted protein which is cleaved by a protease endogenous to the mucocyst pre-domain; and (b) a desired heterologous soluble polypeptide. When the fusion protein is trafficked to a mucocyst, the mucocyst-targeting polypeptide is proteolytically removed by the endogenous protease, resulting in release of the heterologous soluble polypeptide within the mucocyst. Induction of regulated secretion from the mucocysts results in the discharge of the mucocysts contents, including the heterologous soluble polypeptide. Because the desired heterologous polypeptide is soluble, whereas the extracellular matrix formed by regulated secretion is insoluble, the heterologous polypeptide can be isolated from the matrix by standard techniques.

[00116] In some embodiments, the fusion protein further comprises an endoplasmic reticulum-targeting polypeptide N-terminal to said mucocyst-targeting polypeptide. Thus, the structure of the fusion protein can be, N-terminally to C-terminally,

(a) an endoplasmic reticulum-targeting polypeptide; (b) a mucocyst-targeting polypeptide of a mucocyst-targeted protein which is cleaved by a protease endogenous to the mucocyst pre-domain; and (c) a desired heterologous soluble polypeptide. The endoplasmic reticulum-targeting polypeptide can be cleaved from the fusion protein in the ER, but this is not required if the endoplasmic reticulum-targeting polypeptide does not interfere with mucocyst-targeting, or the cleavage of the mucocyst-targeting polypeptide from the heterologous polypeptide.

[00117] In some embodiments, the mucocyst-targeted protein is a Grl protein, including any of a Grl-1 protein, a Grl-2 protein a Grl-3 protein, a Grl-4 protein, a Grl-5 protein, a Grl-6 protein, a Grl-7 protein, a Grl-8 protein, a Grl-9 protein, and a Grl-10 protein. In these embodiments, the mucocyst-targeting polypeptide is the pro-domain of the Grl protein.

[00118] In other embodiments, the mucocyst-targeted protein is a cortical granule protein other than a Grl. A number of endogenous proteins that traffic to the mucocysts but do not associate with the crystalline structure are known (Haddad *et al.* (2002); Bowman *et al.* (2005a)). For example, the mucocyst-targeting polypeptide Igr1p (for Induced during Granule Regeneration) can be employed (accession number AAL79508). Alternatively, granule tip proteins can be employed (accession numbers ABC75092; AAZ94627) (Bowman et al. (2005a)). Other proteins with a β/γ crystalline domain have been identified and also can be used in the invention, for example, C-terminal crystallin fold containing protein 3p (accession number ABC75094); C-terminal crystallin fold containing protein 4p (accession number ABC75093); C-terminal crystallin fold containing protein 6p (accession number ABC75099); C-terminal crystallin fold containing protein 7p (accession number ABC75098); C-terminal crystallin fold containing protein 8p (accession number ABC75091); C-terminal crystallin fold containing protein 9p (accession number ABC75097); C-terminal crystallin fold containing protein 10p (accession number ABC75096); C-terminal crystallin fold containing protein 11p (accession number: ABC75090); C-terminal crystallin fold containing protein 12p (accession number ABC75095); C-terminal crystallin fold containing protein 13p (accession number ABC75100).

[00119] The endoplasmic reticulum-targeting polypeptide, when present, can be from the same protein as the mucocyst-targeting polypeptide, or it can be heterologous. Indeed, the endoplasmic reticulum-targeting polypeptide can be from any ER-targeted polypeptide, even from different species, as long as it is effective as an ER signal sequence.

In some embodiments, the endoplasmic reticulum-targeting polypeptide is the ER signal sequence or pre-domain of a Grl protein or other granule-associated protein. In other embodiments, it can be a heterologous or exogenous sequence, such as the 22 amino acid signal peptide derived from the immobilization antigen variant B protein of *Ichthyophthirius multifiliis*, which has been shown to be functional in *Tetrahymena*.

[00120] In Figure 3, Construct #1 illustrates a construct is which, N-terminally to C-terminally, a endoplasmic reticulum-targeting polypeptide (pre) is fused to mucocyst-targeting polypeptide (pro), which is fused to the desired heterologous soluble polypeptide (HSP). When the fusion protein is synthesized, it is trafficked to the ER due to the endoplasmic reticulum-targeting polypeptide, which is typically (but not necessarily) removed by proteolytic processing in the ER. The mucocyst-targeting polypeptide causes the fusion protein (with or without the endoplasmic reticulum-targeting polypeptide) to be trafficked to the secretory granules, where it is cleaved by a sequence-specific protease endogenous to the mucocyst. This results in the release of the heterologous soluble polypeptide (HSP) within the mucocyst.

[00121] In a second series of embodiments, a first fusion protein comprises, N-terminally to C-terminally, (a) a desired heterologous soluble polypeptide; and (b) at least a mucocyst-targeting polypeptide of a mucocyst-targeted protein. The first fusion protein may optionally include additional sequences from the mucocyst-targeted protein extending C-terminally from the mucocyst-targeting polypeptide. In such embodiments, when the first fusion protein is trafficked to a mucocyst, the mucocyst-targeting polypeptide is proteolytically cleaved by an endogenous first protease, such that the additional C-terminal sequences are removed, but the heterologous polypeptide remains fused to the mucocyst-targeting polypeptide, thereby producing a second fusion protein . Induction of regulated secretion from the mucocysts results in the discharge of the mucocyst contents, including the second fusion protein.

[00122] In some embodiments, the first fusion protein further comprises an endoplasmic reticulum-targeting polypeptide N-terminal to said mucocyst-targeting polypeptide. Thus, the structure of the fusion protein can be, N-terminally to C-terminally, (a) an endoplasmic reticulum-targeting polypeptide; (b) a desired heterologous soluble polypeptide; and (c) at least a mucocyst-targeting polypeptide of a mucocyst-targeted protein. The first fusion protein may optionally include additional sequences from the mucocyst-targeted protein extending C-terminally from the mucocyst-targeting polypeptide, including

the entire mucocyst-targeted protein sequence. In such embodiments, when the first fusion protein is trafficked to a mucocyst, the mucocyst-targeting polypeptide is proteolytically cleaved by an endogenous first protease, such that the additional C-terminal sequences are removed, but the heterologous polypeptide remains fused to the mucocyst-targeting polypeptide, thereby producing a second fusion protein . Induction of regulated secretion from the mucocysts results in the discharge of the mucocyst contents, including the second fusion protein.. The endoplasmic reticulum-targeting polypeptide can be cleaved from the first fusion protein in the ER, but this is not required if the endoplasmic reticulum-targeting polypeptide does not interfere with mucocyst-targeting, or the cleavage of the mucocyst-targeting polypeptide from the heterologous polypeptide.

[00123] In some embodiments, the first fusion protein further comprises a second protease cleavage site between the heterologous polypeptide and the mucocyst-targeting polypeptide of said mucocyst-targeted protein. After inducing regulated secretion of the mucocysts, the extracellular matrix (including the second fusion protein) can be contacted with the second protease to cleave the second cleavage site and separate the mucocyst-targeting polypeptide from the heterologous polypeptide. Alternatively, the second fusion protein can be partially or completely separated from the extracellular matrix, and then the second fusion protein can be contacted with the second protease to cleave the second cleavage site and separate the mucocyst-targeting polypeptide from the heterologous polypeptide. In either case, the desired heterologous polypeptide may then be further purified.

[00124] In Figure 3, Construct #2 illustrates a construct is which, N-terminally to C-terminally, a endoplasmic reticulum-targeting polypeptide (pre) is fused to a desired heterologous soluble polypeptide (HSP), which is fused to a cleavage site (CS), which is fused to a mucocyst-targeting polypeptide (pro), which is fused to additional sequences from the mucocyst-targeted protein (*e.g.*, the entire mature protein sequence). When the fusion protein is synthesized, it is trafficked to the ER due to the endoplasmic reticulum-targeting polypeptide, which is typically (but not necessarily) removed by proteolytic processing in the ER. The mucocyst-targeting polypeptide causes the fusion protein (with or without the endoplasmic reticulum-targeting polypeptide) to be trafficked to the secretory granules, where it is cleaved by a sequence-specific protease endogenous to the mucocyst. This results in the release of a second fusion protein, comprising the heterologous soluble polypeptide (HSP), cleavage site (CS) and mucocyst-targeting polypeptide (pre) within the mucocyst.

Reaction of this fusion protein with a protease specific for the cleavage site (CS) results in release of the desired heterologous soluble polypeptide (HSP).

[00125] In a third series of embodiments, a first fusion protein comprises, N-terminally to C-terminally, (a) an endogenous soluble mucocyst-targeted polypeptide; (c) a sequence-specific protease cleavage site; and (c) a desired heterologous soluble polypeptide. In these embodiments, the soluble endogenous mucocyst polypeptide is normally trafficked to the mucocysts by virtue of its endogenous ER and mucocyst signal sequences. The heterologous protein is also trafficked to the mucocyst by virtue of its fusion to the soluble endogenous mucocyst polypeptide. Induction of regulated secretion from the mucocysts results in the discharge of the mucocyst contents, including the fusion protein. After inducing regulated secretion of the mucocysts, the extracellular matrix (including the fusion protein) can be contacted with a protease to cleave the cleavage site and separate the endogenous soluble mucocyst protein from the heterologous soluble polypeptide. Alternatively, the fusion protein can be partially or completely separated from the extracellular matrix, and then the fusion protein can be contacted with the protease to cleave the cleavage site and separate the endogenous soluble mucocyst polypeptide from the heterologous soluble polypeptide. In either case, the desired heterologous soluble polypeptide may then be further purified.

[00126] In some embodiments, the endogenous soluble mucocyst-targeted protein is an Igr protein. For example, Igr1p is roughly 40-fold more soluble than the Grls and, therefore, can be fused with a desired heterologous soluble polypeptide such that the fusion protein will traffic to the granules and remain soluble as well.

[00127] In some embodiments, the fusion protein further comprises an endoplasmic reticulum-targeting polypeptide N-terminal to the endogenous soluble mucocyst-targeted polypeptide. The endoplasmic reticulum-targeting polypeptide, when present, can be from the same protein as the endogenous soluble mucocyst-targeted polypeptide, or it can be heterologous. Indeed, the endoplasmic reticulum-targeting polypeptide can be from any ER-targeted polypeptide, even from different species, as long as it is effective as an ER signal sequence. In some embodiments, the endoplasmic reticulum-targeting polypeptide is the ER signal sequence or pre-domain of a Grl protein or other granule-associated protein. In other embodiments, it can be a heterologous or exogenous sequence, such as the 22 amino acid signal peptide derived from the immobilization antigen variant B protein of *Ichthyophthirius multifiliis*, which has been shown to be functional in

Tetrahymena. In these embodiments, the endoplasmic reticulum-targeting polypeptide can be cleaved from the fusion protein by endogenous processing in the ER.

[00128] In Figure 3, Construct #3 illustrates a construct is which, N-terminally to C-terminally, an endoplasmic reticulum targeting polypeptide (pre) is fused to a soluble polypeptide (SP) endogenous to the mucocyst, which is fused to a protease cleavage site (CS), which is fused to a heterologous polypeptide (HSP). When the fusion protein is trafficked to a mucocyst, the pre-domain is proteolytically removed, resulting in release of the fusion of the endogenous soluble polypeptide (SP), cleavage site (CS), and heterologous polypeptide (HSP). After regulated secretion, this fusion can be treated with the corresponding protease to release the heterologous polypeptide (HSP), before or after separating it from the extracellular matrix formed by mucocyst discharge.

[00129] Genetic constructs encoding such fusion proteins can readily be prepared by one of skill in the art based upon the universal genetic code, and optionally employing the codon preferences characteristic of the ciliate host. See, Larsen *et al.* (1999); Wuitschick and Karrer (2000); Eisen *et al.* (2006); and Wuitschick and Karrer (1999).

[00130] The genetic constructs can be designed to include a cleavable linker such as protease cleavage site, self-cleaving intein sequence, or flexible linker sequence between the mucocyst targeting sequence(s) and the heterologous polypeptide, and/or may be designed to include additional sequences useful for purification of the fusion protein (*e.g.*, poly-His or epitope tags for affinity or immuno purification).

[00131] The sequences encoding the fusion protein can be introduced into the cells on expression plasmids, or can be stably integrated into the protist genome (*e.g.*, by homologous recombination, retroviral insertion). When integrated into the genome, the fusion protein sequences can replace (in whole or in part) the endogenous sequences encoding the corresponding mucocyst protein, or can be inserted at a separate genomic location. Targeting sequences useful for secretion of foreign proteins in *Tetrahymena spp.* are described in (Clark *et al.* (2001)).

[00132] The nucleic acid sequences can be cloned using standard cloning procedures in the art, as described by Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Springs Laboratory, Cold Springs Harbor, N.Y. (1989). For example, chimeric genes encoding the fusion proteins can be generated by linking coding regions of genes for the heterologous polypeptides to endogenous mucocyst targeting

sequences (or mucocyst protein fragments or entire mucocyst proteins) either synthetically (Lin *et al.* (2002)), or by PCR using serial overlap extension. The resulting constructs can then introduced into standard plasmid DNA vectors (*e.g.*, TOPO, BlueScript, etc.) for amplification in *E. coli* by chemical transformation, electroporation or any other method known in the art.

[00133] Ciliates Useful in the Invention

[00134] The invention may be practiced with a variety of different ciliates which include secretory granules called mucocysts. Heterologous polypeptides can be targeted to these secretory granules by encoding fusion proteins of the desired heterologous polypeptide and an appropriate targeting sequence. After exposing the ciliate to a secretory stimulus that causes the mucocysts to discharge their contents to the extracellular environment, the heterologous polypeptide can be recovered from the resulting matrix and medium.

[00135] The free-living ciliate protists are a large and diverse phylum (*Ciliata*) whose members display a structural and functional complexity comparable to that of higher metazoa (Fankel (2000); Turkewitz *et al.* (2002)), and include over 7,000 species with 11 major subdivisions. Tetrahymenids and Paramecium belong to the Oligohymenophoreans. Ciliates that include mucocysts useful in the invention include *Tetrahymena* species such as *Tetrahymena thermophila* and *Tetrahymena pyriformis*. *Paramecium* has dense core granules but does not secrete a proteinaceous gel. Both *Tetrahymena thermophila* and *Tetrahymena pyriformis* produce mucocysts, and both secrete a proteinaceous gel.

[00136] *Tetrahymena spp.* are amenable to genetic manipulation, can be grown on a large scale and have a doubling time of 1.5-3 hrs. Unlike *T. thermophila*, which has an optimal growth temperature of 35°C, the optimal growth temperature for *T. pyriformis* is lower (maximal growth temperature of 34°C). Cells reach high-density in a short time on a variety of inexpensive media and can be expanded for growth in bioreactors up to several thousand liters in size (Hellenbroich *et al.* (1999); de Coninck *et al.* (2000)). Methods for transformation, along with robust, inducible promoters for driving high-level gene expression have recently been described for this system (Bruns and Cassidy-Hanley (2000); Gaertig and Kapler (2000); Shang *et al.* (2002); Boldrin *et al.* (2006)).

[00137] *Tetrahymena spp.* devote a large part of their metabolism to membrane protein production due to the hundreds of cilia that extend from its surface (Williams *et al.* (1980)). Additionally, *Tetrahymena spp.* lack a cell wall and display high-mannose N-glycan

protein modifications that lack branched, immunogenic structures (Taniguchi *et al.* (1985); Becker and Rusing (2003); Weide *et al.* (2006)). Glycosylation patterns of secreted proteins in *Tetrahymena spp.* are uniform and consist of high-mannose N-glycan structures comprising $Man_3GlycNac_2$ core N-glycans similar to those which are produced in the endoplasmic reticulum of mammalian cells.

[00138] This glycosylation pattern is unlike the glycosylation pattern produced in other microbial systems. For example, such glycosylation is non-existent in bacteria, and is highly branched and immunogenic in fungi.

[00139] Vectors

[00140] Heterologous nucleic acids can be introduced into the ciliate host on an expression vector that is capable of integrating into the host's genome. For example, expression vectors capable of homologous recombination with a highly expressed gene that is endogenous to the protozoan host, such as a P-tubulin gene are known in the art. Alternatively, a heterologous nucleic acid transformed into a ciliate can be maintained extrachromosomally on an autonomous plasmid.

[00141] Expression vectors useful for transforming ciliates in accordance with the methods described herein include but are not limited to replacement vectors, rDNA vectors, and rDNA-based vectors. Replacement vectors accomplish DNA-mediated transformation by replacing or altering endogenous genes using homologous recombination. Integration of the heterologous nucleic acid into the host's genome at the targeted site is accomplished via homologous recombination involving a double crossover event with the vector containing the heterologous nucleic acid. An example of an expression vector useful for genomic incorporation of a heterologous nucleic acid by replacement is one that includes a heterologous coding sequence flanked by portions of the endogenous BTU1 gene of *Tetrahymena thermophila*.

[00142] A replacement vector can include a 5' region, followed by a heterologous coding region, followed by a 3' region, wherein at least a portion of each of the 5' and 3' regions is complementary to 5' and 3' regions on an endogenous gene of the host, to allow for genomic integration of the heterologous coding region via homologous recombination. The 5' and 3' regions of the vector can also comprise regulatory elements, such as a promoter and a terminator. The necessary regulatory elements can also be supplied by the endogenous gene into which the heterologous coding region integrates. Suitable regulatory regions

include, but are not limited to promoters, termination sequences, signal peptides and proprotein domains involved in the expression and secretion of proteins. For example, such regulatory elements can provide efficient heterologous expression of proteins in *Tetrahymena spp.* under control of promoters and/or terminators which are derived from genes in *Tetrahymena ssp.* Such vectors can comprise naturally occurring promoters and/or terminators from proteins secreted at a high level in *Tetrahymena ssp.* The expression of recombinant polypeptides in *Tetrahymena spp.* can be driven by strong promoters, pre/pro sequences and terminators. In one embodiment, the promoters and/or terminators can be selected from proteins secreted at a high level independent of the cell-cycle in *Tetrahymena spp.* (US Patent Application 2006/0127973; WO2003/078566). Inducible promoters from *Tetrahymena spp.* genes have also been described that allow robust expression of foreign genes. For example, heat-inducible promoters of the heat shock protein family of the ciliate *Tetrahymena spp.* are also suitable for use with the methods described herein. Suitable heat shock promoters from *Tetrahymena spp.* are known in the art (see WO2007/006812).

[00143] Methods for creating mitotically stable *Tetrahymena spp.* transformants, for example, by integration of a heterologous gene by homologous DNA recombination, are known in the art. Methods for generating *Tetrahymena spp.* having targeted gene knockouts by homologous DNA recombination are also known in the art (Bruns and Cassidy-Hanley (2000); Hai *et al.* (2000) 514-531; Gaertig *et al.* (1999); Cassidy-Hanley *et al.* (1997)). The somatic macronucleus or the generative micronucleus can be transformed in alternation. For example, sterile transformants, which may provide improved safety parameters, can be obtained with macronucleus transformation.

[00144] Expression vectors can also be maintained extrachromosomally in the ciliates. An expression vector maintained as an extrachromosomal element can be a rDNA-based vector containing an ori from *Tetrahymena spp.* rDNA, which is known to support extrachromosomal replication. Such a vector can further comprise a 5' regulatory region from an endogenous *Tetrahymena spp.* gene containing a promoter region operably linked to the heterologous coding region and, optionally, a 3' regulatory region from the same or a different *Tetrahymena spp.* gene. For example, regulatory regions from ciliate genes in such vectors can include, but are not limited to, regulatory regions from genes such as HHFI, rpl29, BTU1, BTU2, SerH3, and actin.

[00145] There are a number of suitable vectors suitable for transformation of ciliates known in the art. For example, *Tetrahymena spp.* can be transformed with an rDNA

vector (Tondravi and Yao (1986); Yu and Blackburn (1989)). The shuttle vector pXS76 allows insertion of transgenes downstream of a cadmium-inducible promoter from the *MTT1* metallothionein gene of *T. thermophila* via homologous recombination and selection in paromomycin. Alternatively, inserts can be introduced into high copy number ribosomal DNA vectors (such as pD5H8) under control of the cadmium-inducible *MTT1* promoter. The pD5H8 vector takes advantage of a biological feature of *Tetrahymena spp.* in which the ribosomal cistrons become amplified to extraordinarily high copy numbers following conjugation. An rDNA-based vector can be a circular vector that contains a 5' non-translated sequence comprising two or more *ori* sequences from *Tetrahymena spp.* rDNA. A nucleic acid fragment containing a heterologous coding region, for example a selectable marker or transgene, can also be added to the vector. The vector can further comprise a 5' untranslated region of a *Tetrahymena spp.* gene and a 3' untranslated region of a *Tetrahymena spp.* gene, inserted upstream and downstream of the selectable marker and/or the transgene. Methods for transformation, along with robust, inducible promoters for driving high-level gene expression have recently been described for this system (Bruns and Cassidy-Hanley (2000); Gaertig and Kapler (2000); Shang *et al.* (2002); Boldrin *et al.* (2006)).

[00146] Sequence variations within the origins of replication of rDNA from wild-type B- and C3- strains of *T. thermophila* convey a replicative advantage to the C3- form in B/C3 heterozygotes. Although both B- and C3- forms of rDNA are initially present in the macronucleus in approximately equal amounts, within 30 fissions only the C3 variant remains (Pan *et al.* (1982); Orias *et al.* (1988)). pIC19-based shuttle vectors containing the C3 origin of replication have been used as high-copy number vectors for the delivery of foreign DNA to *Tetrahymena spp.* (Yu and Blackburn (1989)) (Figure 5).

[00147] Although such vectors can become unstable and be lost within about 50 to about 80 generations, micronuclear versions of the C3 rDNA is accurately processed (to form a palindrome) following introduction into *T. thermophila* B cell lines. The micronuclear version is maintained as a stable linear chromosome over many generations (Bruns *et al.* (1985)). Functional transgenes can be inserted into the 3'-nontranscribed spacer (3'-NTS) of such vectors with no effect on rDNA processing. Within 6-10 generations, recombinant molecules can comprise 50-100% of the total rDNA complement, with as many as 18,000 copies of the transgene per cell (Blomberg *et al.* (1997)). The use of this approach enables an increase in the number of cloned genes in transformed cell lines by orders of magnitude and leads to increased expression at the protein level. For example, the use of rDNA-based

vectors in combination with the *MTT1* promoter can be used to drive expression of the endogenous granule lattice protein Grl1p to approximately 20% of total cell protein (Lin *et al.* (2002)). Similarly, pD5H8 rDNA-based vectors (Blomberg *et al.* (1997)) can be used to boost expression of proteins by at least 3-10 fold compared with transformants in which respective transgenes are integrated at somatic gene loci. Other vectors suitable for use with the methods described here include vectors comprising a ribosomal DNA sequence. Such vectors can replicate at high copy numbers and can be used to deliver a heterologous DNA sequence to *Tetrahymena spp.* for purposes of RNA expression.

[00148] Transformation.

[00149] Genes can be introduced into ciliates using established protocols or any method known to one skilled in the art. Transformation of ciliates can be achieved by microinjection (Tondravi and Yao (1986)), electroporation (Gaertig and Gorovsky (1992)), or biolistically (Cassidy-Hanley *et al.* (1997)).

[00150] Thus, in some embodiments, ciliate cells can be transformed with a chimeric gene by particle bombardment (also known as biolistic transformation) (Cassidy-Hanley *et al.* (1997)). Particle bombardment transformation can be achieved by several ways. For example, inert or biologically active particles can be propelled at cells under conditions effective to penetrate the outer surface of the cell and to be incorporated within the interior thereof. When inert particles are utilized, the vector can be introduced into the cell by coating the particles with the vector containing the chimeric gene. Alternatively, the target cell can be surrounded by the vector so that the vector is carried into the cell by the wake of the particle. Other variations of particle bombardment, now known or hereafter developed, can also be used.

[00151] Microcarrier bombardment can also be used to transform ciliate cells by means of DNA-loaded gold particles (US Pat. No. 6,087,124; European Pat. EP 847 444; WO 1998/001572). In this approach, microcarrier bombardment with DNA-coated gold is used as a means of introducing foreign genes into ciliates. In one embodiment, microcarrier bombardment can be used to transform ciliates and introduce genes into the (germline) micronucleus

[00152] Methods for selection of transformed cells harboring foreign genes are known in the art. For example, the vector can further comprise a selectable cassette marker to permit selection for transformed cells (*e.g.*, a neo 2 cassette) (Gaertig *et al.* (1994)).

Selection of transformants can be achieved by growing the cultured ciliates in a medium which allows only the transformants to survive. Suitable selection agents include antibiotics which will kill most all non-transformants but allow transformants (which also possess an antibiotic resistance gene) to survive. A number of antibiotic-resistance markers are known in the art. Any known antibiotic-resistance marker can be used to transform and select transformed host cells in accordance with the present invention. For example, selection of the transformants can be performed by means of a resistance marker such as a point mutation in the 17s rDNA, which confers resistance to paromomycin, can allow for selection of rDNA transformants (Spangler and Blackburn (1985); Bruns *et al.* (1985)). Other methods include the use of a mutant cell line that allows targeting of genes to the beta tubulin-1 locus of *T. thermophila* by homologous recombination, and allows efficient selection of transformed cell lines by growth in the microtubule-stabilizing agent (taxol) (U.S. Pat. No. 6,846,481). Another method for selection of transformed cells harboring foreign genes is to insert full length coding regions into the pD5HA vector (Cowan *et al.* (2005)). In this method, transcription is driven by the inducible MTT1 promoter. Once cells have been transformed with the pD5HA vector selection of positive transformants is determined by paromomycin resistance (*i.e.*, cell growth in media containing the drug). Presence of the transgene is then verified by PCR and then induced with cadmium chloride to over-express the recombinant gene product.

[00153] Many other selectable marker systems are known in the art. Selectable marker genes that confer resistance or tolerance to a normally toxic selection agent allow only successfully transfected cells to survive in the presence of the selection agent, and are referred to as positive selectable markers. Examples of positive selectable marker genes and their corresponding selection agents are: aminoglycoside phosphotransferase (APH) and G418; dihydrofolate reductase (DHFR) and methotrexate (Mtx); hygromycin-B-phosphotransferase (HPH) and hygromycin-B; xanthine-guanine phosphoribosyltransferase (XGPRT) and mycophenolic acid; and adenosine deaminase (ADA) and 9-β-D-xylofuranosyl adenine (Xyl-A). In another example of a positive selectable marker system, thymidine kinase (TK) and aminopterin (included, *e.g.*, in hypoxanthine-aminopterin-thymidine (HAT) medium) can be used in cells that are initially thymidine kinase deficient (tk^-). The aminopterin will normally kill tk^- cells and, therefore, only successful TK transfectants will survive. Selectable marker genes that confer sensitivity or susceptibility to a normally non-toxic selection agent cause only successfully transfected cells to die in the presence of the

selection agent, and are referred to as negative selectable markers. An example of a negative selectable marker system is thymidine kinase (TK) and gancyclovir. Phenotypic selectable marker genes permit selection based upon morphological or biochemical traits rather than cell death or survival. In some cases, the phenotypic marker is detectable only in the presence of an additional selection agent. An example of a phenotypic selectable marker system is β-galactosidase (*lacZ*) and X-gal.

[00154] Isolation of Desired Polypeptides from the Mucocyst Matrix.

[00155] In one aspect, the invention provides methods for protein purification from the extracellular matrix formed by the discharge of mucocysts. Because heterologous polypeptides targeted to the mucocyst compartment will be associated within the matrix, the invention provides matrix-based purification strategies. Advantageously, the matrix can be used for rapid purification of recombinant polypeptides associated with it.

[00156] Proteins within the gel matrix can be separated from cellular constituents by low-speed centrifugation (See Turkewitz *et al.* (2000)). Any other method known in the art suitable for separating intact cells, from the discharged material, including, but not limited to filtration harvesting using an appropriately selected mesh, can also be used in conjunction with the methods described herein. After isolation of the matrix, the desired heterologous polypeptide can be liberated from the secreted matrix gel. Methods for liberation of the protein can include chemical methods (*e.g.*, high salt concentrations) and/or enzymatic methods (*e.g.*, site-specific proteases).

[00157] Proteins can also be isolated in intact secretory granules. For example, the use of an exocytosis-defective mutant, MN173, of *T. thermophila* where granules accumulate in the cytoplasm has been described for such purposes (Melia *et al.* (1998)).

[00158] Heterologous Polypeptides.

[00159] Suitable heterologous polypeptides for use with these methods include, but are not limited to, antibodies, antibody fragments, cytokines, growth factors, protein kinases, proteases, protein hormones or any fragment thereof. Similarly, the methods described herein are suitable for the production of specialty proteins. The use of such specialty proteins can include, but is not limited to, prototype vaccines for animal model studies, structural studies, or as therapeutic proteins. For example, quantities of antigens can be produced according to the methods described herein.

[00160] Mucus as a Vaccine Delivery Vehicle

[00161] In the case where antigens are produced according the methods described herein, the mucus can serve as a vehicle for the delivery of subunit vaccine antigens to humans and other vertebrates in a highly potent form. In these embodiments, the antigens are not purified from the mucus matrix. Rather, the mucus containing one or more heterologous antigens can serve as the vaccine. The potency of this material can be attributed to several unique properties of the mucus. First, the material stored by dense core granules forms a crystalline array. The crystalline nature of this material is retained following mucocyst discharge as an expanded (hydrated) proteinaceous gel. Proteins within the mucus are therefore present in a highly repetitive form with a molecular spacing that is advantageous for cross-linking the immunoglobulin receptor on B-cells. Lateral clustering of Ig receptors on the B-cell membrane provides a strong signal for these cells to divide and produce large quantities of antibodies. In this way, the mucus can induce B-cell responses to co-administered antigens without the need for T-cell help. Although it derives from an entirely different source, the material comprising the mucus has similar properties to virus-like particles but can be made in large volumes at very low cost.

[00162] In addition to enhancing antibody production by B-cells, the mucus is a particulate substance, and as such, can be avidly phagocytosed by professional antigen-presenting cells that are critical to the stimulation of cell-mediated immunity by T-lymphocytes.

[00163] Mucus can be engineered to contain more than a single antigen in the same vaccine formulation by co-expressing multiple fusion proteins comprising different antigens in one ciliate cell. Such formulations would be useful in the manufacture of multivalent vaccines against different strains of the same pathogen, or combination vaccines that target completely unrelated pathogens.

[00164] In addition to antigens, the mucus can also be engineered to contain immunostimulatory substances that will enhance the immune response to co-administered antigens. Professional antigen-presenting cells are equipped with pattern-recognition receptors that scan the environment for pathogen-associated molecules, and other so-called "danger" signals that alert them to the presence of a threat. The danger signals include proteins, carbohydrates, lipids, nucleic acids and various small molecules such as uric acid. Once recognized by a pattern recognition receptor, these molecules convey activating signals

to professional antigen presenting cells that enhance their ability to promote T-cell development and proliferation. Many such immunostimulatory substances are known in the art. Examples include, but are not limited to, bacterial flagellin, pathogen-associated glycolipid anchors, double-stranded RNA, bacterial DNA, CpG oligonucleotides, profilin, complement component C3d, heat shock proteins, high mobility group proteins, and others. In the case where these substances are proteins, they can be co-expressed with immunogenic peptides comprising vaccine antigens and co-administered with the mucus as highly potent vaccines.

[00165] Immunostimulatory substances can also be incorporated into mucus that contains heterologous vaccine antigen(s) by non-specific adsorption, or by specific binding to a fusion protein that is a receptor for the immunostimulatory substances and is co-expressed with the vaccine antigen(s) in the mucus.

[00166] The following examples illustrate some preferred modes of practicing the present invention, but are not intended to limit the scope of the claimed invention. Alternative materials and methods may be utilized to obtain similar results.

EXAMPLES

Example 1. Targeting Heterologous Proteins to Mucocysts

[00167] The amino acid sequence for green fluorescent protein (GFP) can be linked to the granule lattice proteins of *Tetrahymena spp.*, namely (*e.g.*, Grl1p), and the resulting Grl1p:GFP chimera traffics to mucocysts *in vivo* (Bowman et al (2005), *Traffic* 6:303-323). This demonstrated that the targeting sequences of the granule-lattice proteins can be used to localize heterologous polypeptides to mucocysts. This result was confirmed using a vaccine antigen from avian influenza virus and a single-chain antibody fragment against anthrax PA toxin linked to the C-terminus of Grl1p (Figures 4-6). As shown herein, (1) such proteins localize to cortical secretory granules, (2) mucocysts that contain such proteins can be functional and discharge their contents, (3) proteins linked to Grl1p associate with the mucocyst gel following granule discharge and (4) proteins of interest could be released from the mucocyst gel and recovered in a purified form

Example 2. Targeting and Purification of Recombinant Proteins

[00168] *Tetrahymena thermophila* cells can be used as a platform for overexpression of recombinant polypeptides (Figure 1). *Tetrahymena spp.* cells grow rapidly

to high cell density in inexpensive media. *Tetrahymena spp.* also accumulates endogenous protein in cortical secretory granules, or mucocysts, which discharge their contents in a stimulus-dependent fashion in response to a variety of secretogogues. Proteins stored in mucocysts can self-associate and form an insoluble proteinaceous gel when released from cells (Figure 2). In one aspect, the methods describe herein relate to the finding that this gel can serve as a matrix for the purification of recombinant polypeptides. In one embodiment, the gel provides a means for separating recombinant gene products from the bulk of contaminating cellular proteins.

[00169] *Tetrahymena spp.,* like other ciliates, has two, functionally distinct nuclei: a polyploid macronucleus that is transcriptionally active, and a diploid micronucleus that is transcriptionally silent and functions only in sexual conjugation. When cells mate, the old macronucleus degenerates and is replaced by a new macronucleus that develops from one of several post-zygotic micronuclei. Macronuclear development is accompanied by a spectacular increase in ribosomal DNA (rDNA) copy number. The two rDNA alleles within the micronuclear genome become excised from chromosome 1, and form unique 21 kb palindromic chromosomes that become amplified roughly 9,000 fold.

[00170] Granule lattice proteins are made as preproproteins, with their pre-domains acting as standard signal sequences for ER translocation, and their pro- domains directing vesicle trafficking and maturation within the granules. Propeptides are cleaved from Grls through the action of, as yet unidentified, proteolytic processing enzymes. Thus, in one embodiment, the methods described herein provide a method for purification of a heterologous polypeptide covalently linked to one of more mucocyst proteins secreted into the insoluble gel of a *Tetrahymena spp.* cell. Genetic engineering techniques can be used to covalently link heterologous polypeptides of interest to one or more mucocyst proteins. In one embodiment, the mucocyst protein linked to the heterologous polypeptide can be a protein normally secreted into the gel, such as a granule lattice protein (Grls). For example, a heterologous polypeptide linked C-terminally to a newly synthesized Grl will traffic to mucocysts and be processed into mature Grl linked N-terminally to the heterologous polypeptide partner.

[00171] Such heterologous polypeptides can be harvested upon secretion from the cell. In one embodiment, heterologous polypeptides, such as linked heterologous polypeptides described herein, will associate with the insoluble mucus via their Grl partners upon secretion from the cell and can be harvested by low speed centrifugation or filtration.

For example, such methods can be used for the production and harvesting of single-chain antibody fragments against anthrax PA toxin, and the H5 hemagglutinin of avian influenza virus (Figures 4-6).

[00172] The methods described herein also provide for the introduction of one or more site-specific protease cleavage sites or self-cleaving inteins between the mature Grl sequence and the heterologous polypeptide of interest. When such a site-specific protease cleavage site is introduced between the mature Grl sequence and the heterologous polypeptide of interest, the heterologous polypeptide can be separated from the matrix by treatment of the harvested gel with a site-specific protease.

[00173] Similarly, when a self-cleaving intein is introduced between the mature Grl sequence and the heterologous polypeptide of interest, the heterologous polypeptide can be separated from the matrix by the introduction of conditions that lead to intein cleavage (*e.g.*, treatment with disulfide reducing agents) (Figure 7). Such treatments separate heterologous polypeptides of interest from the gel matrix and permit isolation of the desired proteins in a highly purified form following low-speed centrifugation and/or filtration to remove the insoluble components of the gel.

Example 3: Mucocyst Targeting

[00174] Chimeric genes were synthesized by GenScript Inc (Piscatawy, NJ). Restriction enzymes were purchased from New England Biolabs. Tetrahymena cells were cultured in NEFF medium (0.25% proteose peptone, 0.25% yeast extract, 0.55% glucose, 33 μM $FeCl_3$) supplemented, when required, with paromomycin at a final concentration of 100 μg/ml. All medium components were acquired from VWR. For Biolistic transformations DNAdel^{TM} S550d gold carrier particle suspension was purchased from Seashell Technology and filter paper from Whatman. Western analysis was carried out with a conformation specific neutralizing mouse monoclonal antibody, 5C5. Anti-mouse horse-radish peroxidase (HRP)-conjugated secondary antibodies was purchased from Bio-Rad. Insect cell-derived H5N1 hemagglutinin was obtained from Protein Sciences. For induction of regulated secretion Dibucaine was purchased from Sigma-Aldrich and Protease Inhibitor Cocktail from Roche.

[00175] **Expression construct design, synthesis and cloning:** The fusion construct was comprised of a truncated form of the avian influenza H5N1 hemagglutinin lacking the transmembrane domain (H5ΔTMD) fused in-frame to the Tetrahymena Grl1

protein comprising the Pro domain and mature Grl1 amino acid sequence ($H5\Delta TMD^{ProGrl1}$, Figure 8 Panel A). The genes encoding $H5\Delta TMD^{ProGrl1}$ was chemically synthesized with flanking BamHI and SacI restriction sites and subsequently cloned into the same restriction sites of a Tetrahymena somatic expression vector, pXS76. Transcription of the transgene is under control of a robust cadmium-inducible promoter from the metallothionein-1 (*MTT1*) gene of *Tetrahymena thermophila.* The expression construct comprising MTT1 promoter, transgene, MTT1 terminator and a neomycin resistance cassette were transferred, *en masse*, as a NotI fragment into a high-copy rDNA vector, pD5H8 and introduced into conjugating *Tetrahymena thermophila* strains by biolistic transformation.

[00176] Generation of expression strains: B2086 and CU428 *T. thermophila* strains were grown in modified NEFF medium (0.25% proteose peptone, 0.25% yeast extract, 0.55% glucose, 33 mM $FeCl_3$) at 30°C. One hundred ml of each logarithmically growing culture was centrifuged at 1,100 x g for 2 minutes in oil centrifuge tubes, washed in 10 mM Tris pH 7.4 and resuspended in fresh 10 mM Tris pH 7.4 (starvation medium) at a concentration of 200,000 – 250,000 cells/ml. Cells were incubated for 9-18 hours at 30°C. After starvation, B2086 and CU428 cell cultures were counted and cell concentration was readjusted to 200,000 cells/ml. To induce conjugation, 100 ml of each strain were mixed together in a 4 L flask. Four transformations were performed between 9.5 and 10.5 hours post-mixing using a Biolistic PDS-1000/He Particle Delivery System (BIO-RAD). For each transformation, 20 µl of DNAdel™ S550d gold carrier particle suspension were coated with 4 µg of DNA construct according to manufacturer's instructions. Fifty ml of conjugating cells were concentrated to ~1 ml by centrifugation at 1,100 x g in oil centrifuge tubes for 2 minutes. Cells were spread on a round 90 mm hardened paper filter (Whatman, Cat. # 1450-090) pre-wet with 1.5 ml 10 mM Tris pH 7.4 inside a Petri dish. After the bombardment, the filter with the cells was transferred into a 500 ml flask containing 50 ml NEFF medium. The flasks were incubated on a slow shaker for ~20 hours at 30°C. At 30 hours post-mixing, 25 ml NEFF medium containing 300 µg/ml paromomycin was added to the 50 ml of cell culture (final paromomycin concentration, 100 µg/ml). Cells were aliquoted into 96 well microplates (150 µl per well). After 3-4 days, the microplates were examined and 5 µl from each of the wells containing paromomycin-resistant cells were transferred into 150 µl NEFF medium containing 100 µg/ml paromomycin on a master 96 well microplate.

[00177] **Western analysis:** To evaluate H5ΔTMD^{ProGrl1} expression, cultures were grown to ~5 x 10^5 cells/ml and induced for 12 hr with 1 μg/ml of $CdCl_2$. Cells were then harvested and lysed in SDS sample buffer in the absence of reducing agents. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes before Western blotting. Blots were probed with the neutralizing mouse monoclonal antibody, 5C5. This antibody recognizes conformational epitopes on H5 that are destroyed by treatment with disulfide reducing agents. Following incubation in primary antibody, blots were probed with secondary goat anti-mouse IgG coupled to HRP for visualization.

[00178] **Induction of regulated secretion:** Expression strains were grown to a cell density of 5 x 10^5 cells/ml prior to induction with 1.5 μg/ml $CdCl_2$. Cells were harvested 16 h post-induction by centrifugation at 2000 x g for 5 minutes. The cell pellet was re-suspended in Buffer A (40 mM Hepes, 1 mM $CaCl_2$) followed by the addition of Dibucaine to a final concentration of 2 mM to induce mucocyst release. An equal volume of ice-cold Buffer A containing 2X protease inhibitor cocktail (PIC) was added and then the mixture was centrifuged at 5000 x g for 2 minutes to separate supernatant, mucus and cell pellet layers. The mucus layer was harvested and re-suspended in 10 volumes of Buffer A containing 1X PIC and centrifuged once more at 5000 x g for 2 minutes.

[00179] **Immunofluorescence:** Cells were induced to express the chimeric fusion gene, fixed and the recombinant H5ΔTMD^{ProGrl1} localized by confocal microscopy. Immunofluorescence staining was carried out with a 1:50 dilution of mouse anti-hemagglutinin mAb (5C5) followed by rhodamine-tagged goat anti-mouse IgG.

[00180] **Results.** Expression of H5ΔTMD^{ProGrl1} in Tetrahymena was examined by immunofluorescence and Western analysis in both whole cell lysates and harvested mucus as described herein. Figure 8 (Panel B) shows that H5ΔTMD^{ProGrl1} is targeted to cortical secretory granules (mucocysts) as evidenced by the punctate staining pattern at the cell periphery. Additionally, Figure 8 (Panel C) shows that H5ΔTMD^{ProGrl1} is expressed and resolved at a molecular weight >148 kDa indicating the formation of higher order structures of the fusion protein. Such higher order structures are likely a consequence of the H5ΔTMD fusion partner since H5N1 hemagglutinin is known to trimerize. H5ΔTMD^{ProGrl1} is recovered in mucus following induced release of mucocyst contents (Figure 8, Panel C).

Example 4: Mucocyst Targeting

[00181] Materials were as described for Example 3

[00182] Methods. Expression construct design, synthesis and cloning: The fusion construct was comprised of a truncated form of the avian influenza H5N1 hemagglutinin lacking the transmembrane domain (H5ΔTMD) fused in-frame to the PrePro domain of Tetrahymena Grl1 ($H5\Delta TMD^{PrePro}$, Figure 9 Panel A). Generation of expression constructs was carried out as described for Example 3. Generation of expression strains, Western analysis, induction of regulated secretion and immunofluorescence was carried out as described in Example 3.

[00183] Results. Expression of $H5\Delta TMD^{PrePro}$ in Tetrahymena was examined by immunofluorescence and Western analysis in both whole cell lysates and harvested mucus as described herein. Figure 9 (Panel B) shows that $H5\Delta TMD^{PrePro}$ is targeted to cortical secretory granules (mucocysts) as evidenced by the punctate staining pattern at the cell periphery. Additionally, Figure 9 (Panel C) shows that $H5\Delta TMD^{PrePro}$ is expressed and resolved at a molecular weight >148 kDa indicating the formation of higher order structures of the fusion protein. Such higher order structures are likely a consequence of the H5ΔTMD fusion partner since H5N1 hemagglutinin is known to trimerize. $H5\Delta TMD^{PrePro}$ is recovered in mucus following induced release of mucocyst contents (Figure 9, Panel C).

Example 5: Mucocyst Targeting

[00184] Materials. Materials were as described for Example 3

[00185] Expression construct design, synthesis and cloning: The fusion construct was comprised of the Tetrahymena Igr1 gene fused in-frame to a truncated form of the avian influenza H5N1 hemagglutinin lacking the transmembrane domain ($H5\Delta TMD^{Igr1}$, Figure 10 Panel A). Generation of expression constructs was carried out as described for Example 3. Generation of expression strains, Western analysis, induction of regulated secretion and immunofluorescence was carried out as described in Example 3.

[00186] Results. Expression of $H5\Delta TMD^{Igr1}$ in Tetrahymena was examined by immunofluorescence and Western analysis in both whole cell lysates and harvested mucus as described herein. Figure 10 (Panel B) shows that $H5\Delta TMD^{Igr1}$ is targeted to cortical secretory granules (mucocysts) as evidenced by the punctate staining pattern at the cell periphery. Additionally, Figure 10 (Panel C) shows that $H5\Delta TMD^{Igr1}$ is expressed and is recovered in mucus following induced release of mucocyst contents.

Example 6: Mucocyst Targeting

[00187] Materials were as described for Example 3 except that rabbit polyclonal anti-EPO antibody was purchased from Santa Cruz Biotech and HRP conjugated Goat anti-rabbit secondary antibody from Bio-Rad.

[00188] **Expression construct design, synthesis and cloning:** The fusion construct was comprised of the amino acid sequence of feline EPO fused in-frame to the H5N1 hemagglutinin signal peptide at the amino-terminus and to the Tetrahymena Grl1 protein comprising the Pro domain and mature Grl1 amino acid sequence at it's carboxy-terminus ($EPO^{ProGrl1}$, Figure 11 Panel A). Generation of expression constructs was carried out as described for Example 3. Generation of expression strains and induction of regulated secretion was carried out as described in Example 3. Western analysis was carried out using an anti-EPO primary antibody and an anti-rabbit HRP conjugated secondary antibody

[00189] **Results** Expression of $EPO^{ProGrl1}$ in Tetrahymena was examined by Western analysis in both whole cell lysates and harvested mucus as described herein. Figure 11 (Panel B) shows that $EPO^{ProGrl1}$ is expressed and is recovered in mucus following induced release of mucocyst contents.

Example 7: Mucocyst Targeting

[00190] Materials. Materials were as described for Example 3 except HRP conjugated anti-HA antibody was purchased from Roche.

[00191] **Expression construct design, synthesis and cloning:** The fusion construct was comprised of the Tetrahymena Grl1 gene fused in-frame to the gene encoding a single chain antibody fragment (anti-anthrax PA toxin). Separating the Grl1 and scFv genes is DNA encoding, 5' to 3', a TEV protease site, a 6 x His tag and a HA peptide epitope ($scFv^{Grl1}$, Figure 12 Panel A). Generation of expression constructs was carried out as described for Example 3. Generation of expression strains, Western analysis, induction of regulated secretion and immunofluorescence was carried out as described in Example 3 except that anti-HA antibodies were used to carry out Western and immunofluorescence analysis.

[00192] **Results.** Expression of $scFv^{Grl1}$ in Tetrahymena was examined by immunofluorescence and Western analysis in both whole cell lysates and harvested mucus as described herein. Figure 12 (Panel B) shows that $scFv^{Grl1}$ is targeted to cortical secretory granules (mucocysts) as evidenced by the punctate staining pattern at the cell periphery.

Additionally, Figure 12 (Panel C) shows that $scFv^{Grl1}$ is expressed and is recovered in mucus following induced release of mucocyst contents.

[00193] Example 8: Mucocyst Targeting

[00194] Materials. Materials were as described for Example 3 except HRP conjugated anti-HA antibody was purchased from Roche.

[00195] Expression construct design, synthesis and cloning: The fusion construct was comprised of the Tetrahymena Grl4 gene fused in-frame to the gene encoding a fragment (amino acids 159-426) of the malarial pfs48/45 antigen. Immediately downstream of the pfs48/45 sequence is a 6 x His tag followed by the carboxy-terminal domain (amino acids 371-441) of the immobilization antigen variant B protein of *Ichthyophthirius multifiliis*. At the carboxy-terminus of the fusion construct is a HA epitope tag (Figure 13 Panel A, $pfs48/45^{Grl4}$). Generation of expression constructs was carried out as described for Example 3. Generation of expression strains, Western analysis, induction of regulated secretion and immunofluorescence was carried out as described in Example 3 except that Western and immunofluorescence analysis was carried out with an anti-HA antibody.

[00196] Results. Expression of $pfs48/45^{Grl4}$ in Tetrahymena was examined by immunofluorescence and Western analysis in both whole cell lysates and harvested mucus as described herein. Figure 13 (Panel B) shows that $pfs48/45^{Grl4}$ is targeted to cortical secretory granules (mucocysts) as evidenced by the punctate staining pattern at the cell periphery. Additionally, Figure 13 (Panel C) shows that $pfs48/45^{Grl4}$ is expressed and is recovered in mucus following induced release of mucocyst contents. A majority of fusion protein in the mucus resolves at approximately 50 kDa indicating that the prepro-domain of Grl4 has been processed *in vivo*.

[00197] Example 9: Mucocyst Targeting

[00198] Materials were as described for Example 3 except HRP conjugated anti-HA antibody was purchased from Roche.

[00199] Expression construct design, synthesis and cloning: The fusion construct was comprised of the PrePro domain of the Tetrahymena Grl1 gene fused in-frame to the gene encoding a fragment (amino acids 159-426) of the malarial pfs48/45 antigen. Immediately downstream of the pfs48/45 sequence is a 6 x His tag followed by the carboxy-terminal domain (amino acids 371-441) of the immobilization antigen variant B protein of *Ichthyophthirius multifiliis*. At the carboxy-terminus of the fusion construct is a HA epitope

tag (Figure 14 Panel A, pfs48/45^{PrePro}). Generation of expression constructs was carried out as described for Example 3. Generation of expression strains, Western analysis and induction of regulated secretion was carried out as described in Example 3 except that Western analysis was carried out with an anti-HA antibody.

[00200] **Results.** Expression of pfs48/45^{PrePro} in Tetrahymena was examined by Western analysis in both whole cell lysates and harvested mucus as described herein. Figure 14 (Panel B) shows that pfs48/45^{PrePro} is expressed and is recovered in mucus following induced release of mucocyst contents. A majority of fusion protein in the mucus resolves at approximately 64 kDa indicating that the prepro-domain of Grl4 has not been processed *in vivo*.

[00201] **Example 10: Extraction/Purification**

[00202] Materials were as described for Mucocyst Targeting Example 3. In addition TEV protease was purchased from Invitrogen and Ni-NTA affinity resin was purchased from Fisher Scientific. Construction of the H5ΔTMD^{ProGrl1} expression cassette, generation of expression strains, Western analysis and induction of regulated secretion were as described for Mucocyst Targeting Example 3.

[00203] **Extraction of H5ΔTMD^{ProGrl1} from mucus and Purification by Ni-NTA chromatography.** Mucus containing H5ΔTMD^{ProGrl1} was re-suspended in 10X volumes of 5 mM Tris pH 6.9 and incubated overnight at 4°C. The mixture was centrifuged at high-speed (10,000Xg for 30 min) to remove the insoluble matrix material and the supernatant was concentrated 10-fold with a 10 MWCO spin filter. The concentrated sample was treated with TEV protease at 30°C and the mixture passed over a Ni-NTA column. The column was washed with 20 mM Tris-Cl, 50 mM NaCl and 40 mM imidazole and bound protein eluted in the same buffer containing 400 mM imidazole. Elution fraction containing H5ΔTMD were identified by Western analysis, pooled and concentrated 10-fold with a 10 MWCO spin filter prior to analysis by SDS-PAGE.

[00204] **Results.** Dilution of H5ΔTMD^{ProGrl1}-containing mucus with 5 mM Tris pH 6.9 and incubation overnight results in dissociation of H5ΔTMD^{ProGrl1} from the insoluble mucus matrix (Figure 15, Panel A). Furthermore, a majority of soluble protein resolves as monomer by SDS-PAGE indicating that the extraction process results in the *ex vivo* processing of the fusion protein, presumably by endogenous mucocyst associated proteases, leading to the separation of the H5ΔTMD and Grl1 proteins (Figure 15, Panel A). Treatment

of soluble extract with TEV protease leads to conversion of the remaining fusion protein to monomer (Figure 15, Panel B). Soluble H5ΔTMD binds to and elutes from Ni-NTA affinity resin (Figure 15, Panel C) leading to recovery of a purified sample of H5ΔTMD (Figure 15, Panel D).

Example 10: Extraction/Purification Solubilization by protease treatment of mucus.

[00205] Materials were as described for Mucocyst Targeting Example 3. In addition TEV protease was purchased from Invitrogen and Ni-NTA affinity resin was purchased from Roche. Construction of the $scFv^{Grl1}$ expression cassette was as described for Mucocyst Targeting Example 7. Generation of expression strains, Western analysis and induction of regulated secretion were as described for Mucocyst Targeting Example 3.

[00206] **Extraction of $scFv^{Grl1}$ from mucus by direct treatment with TEV protease.** Mucus containing $scFv^{Grl1}$ was harvested as described herein. TEV protease was added directly to mucus at a concentration of 70U/ml mucus and incubated for 2 hours at 30° C. The mixture was centrifuged for 10 minutes at 8000 x g and the soluble supernatant removed and passed over Ni-NTA resin (1 ml bed volume). The Ni-NTA column was washed in buffer containing 50 mM Tris-Cl, pH 8, 500 mM NaCl, 0.1% TX-100 and 20 mM imidazole. Bound protein was eluted in 1 ml fractions in buffer containing 50 mM Tris-Cl, pH 8, 500 mM NaCl, 0.05% TX-100 and 250 mM imidazole. Elution fractions containing soluble scFv were identified by anti-HA Western analysis.

[00207] **Results.** Treatment of mucus containing $scFv^{Grl1}$ directly with TEV protease results in the accumulation of soluble scFv as shown in Figure 16 (Panel A, Lane S). This is presumably due to TEV dependant separation of the scFv and Grl1 fusion partners with the latter remaining associated with insoluble mucus matrix. Following solubilization, scFv is amenable to purification with Ni-NTA affinity chromatography as shown in Figure 16, Panel B.

Example 11. Use of Tetrahymena mucus as a carrier and immune stimulating matrix.

[00208] Chimeric genes were synthesized by GenScript Inc (Piscatawy, NJ). Restriction enzymes were purchased from New England Biolabs. Tetrahymena cells were cultured in NEFF medium (0.25% proteose peptone, 0.25% yeast extract, 0.55% glucose, 33 μM $FeCl_3$) supplemented, when required, with paromomycin at a final concentration of 100 μg/ml. All medium components were acquired from VWR. For Biolistic transformations DNAdel™ S550d gold carrier particle suspension was purchased from Seashell Technology

and filter paper from Whatman. Western analysis was carried out with a conformation specific neutralizing mouse monoclonal antibody, 5C5. Anti-rat and mouse horse-radish peroxidase (HRP)-conjugated secondary antibodies were purchased from Bio-Rad. Insect cell-derived H5N1 hemagglutinin was obtained from Protein Sciences. For mucus-vaccine preparation Dibucaine was purchased from Sigma-Aldrich and Protease Inhibitor Cocktail from Roche.

[00209] Expression construct design, synthesis and cloning: Fusion constructs were comprised of a truncated form of the avian influenza H5N1 hemagglutinin lacking the transmembrane domain (H5ΔTMD) fused in-frame to either the PrePro domain of Tetrahymena Grl1 ($H5\Delta TMD^{PrePro}$, Figure 9A) or a Grl1 protein comprising the Pro domain and mature Grl1 amino acid sequence ($H5\Delta TMD^{ProGrl1}$, Figure 8A). Genes encoding each fusion construct were chemically synthesized with flanking BamHI and SacI restriction sites and subsequently cloned into the same restriction sites of a Tetrahymena somatic expression vector, pXS76. In each case transcription of the transgene is under control of a robust cadmium-inducible promoter from the metallothionein-1 (*MTT1*) gene of *Tetrahymena thermophila.* Expression constructs comprising MTT1 promoter, transgene, MTT1 terminator and a neomycin resistance cassette were transferred, *en masse*, as a NotI fragment into a high-copy rDNA vector, pD5H8 and introduced into conjugating *Tetrahymena thermophila* strains by biolistic transformation.

[00210] Generation of expression strains: B2086 and CU428 *T. thermophila* strains were grown in modified NEFF medium (0.25% proteose peptone, 0.25% yeast extract, 0.55% glucose, 33 mM $FeCl_3$) at 30°C. One hundred ml of each logarithmically growing culture was centrifuged at 1,100 x g for 2 minutes in oil centrifuge tubes, washed in 10 mM Tris pH 7.4 and resuspended in fresh 10 mM Tris pH 7.4 (starvation medium) at a concentration of 200,000 – 250,000 cells/ml. Cells were incubated for 9-18 hours at 30°C. After starvation, B2086 and CU428 cell cultures were counted and cell concentration was readjusted to 200,000 cells/ml. To induce conjugation, 100 ml of each strain were mixed together in a 4 L flask. Four transformations were performed between 9.5 and 10.5 hours post-mixing using a Biolistic PDS-1000/He Particle Delivery System (BIO-RAD). For each transformation, 20 ml of DNAdel™ S550d gold carrier particle suspension were coated with 4 μg of DNA construct according to manufacturer's instructions. Fifty ml of conjugating cells were concentrated to ~1 ml by centrifugation at 1,100 x g in oil centrifuge tubes for 2 minutes. Cells were spread on a round 90 mm hardened paper filter (Whatman, Cat. # 1450-

090) pre-wet with 1.5 ml 10 mM Tris pH 7.4 inside a Petri dish. After the bombardment, the filter with the cells was transferred into a 500 ml flask containing 50 ml NEFF medium. The flasks were incubated on a slow shaker for ~20 hours at 30°C. At 30 hours post-mixing, 25 ml NEFF medium containing 300 μg/ml paromomycin was added to the 50 ml of cell culture (final paromomycin concentration, 100 μg/ml). Cells were aliquoted into 96 well microplates (150 ml per well). After 3-4 days, the microplates were examined and 5 ml from each of the wells containing paromomycin-resistant cells were transferred into 150 ml NEFF medium containing 100 μg/ml paromomycin on a master 96 well microplate.

[00211] Western analysis: To evaluate H5ΔTMD^{PrePro} and H5ΔTMD^{ProGrl1} expression, cultures were grown to ~5 x 10^5 cells/ml and induced for 12 hr with 1 μg/ml of $CdCl_2$. Cells were then harvested and lysed in SDS sample buffer in the absence of reducing agents. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes before Western blotting. Blots were probed with the neutralizing mouse monoclonal antibody, 5C5. This antibody recognizes conformational epitopes on H5 that are destroyed by treatment with disulfide reducing agents. Following incubation in primary antibody, blots were probed with secondary goat anti-mouse IgG coupled to HRP for visualization.

[00212] Preparation of mucus-based vaccine formulation: Expression strains were grown to a cell density of 5 x 10^5 cells/ml prior to induction with 1.5 μg/ml $CdCl_2$. Cells were harvested 16 h post-induction by centrifugation at 2000 x g for 5 minutes. The cell pellet was re-suspended in Buffer A (40 mM Hepes, 1 mM $CaCl_2$) followed by the addition of Dibucaine to a final concentration of 2 mM to induce mucocyst release. An equal volume of ice-cold Buffer A containing 2X protease inhibitor cocktail (PIC) was added and then the mixture was centrifuged at 5000 x g for 2 minutes to separate supernatant, mucus and cell pellet layers. The mucus layer was harvested and re-suspended in 10 volumes of Buffer A containing 1X PIC and centrifuged once more at 5000 x g for 2 minutes. The mucus was re-suspended in Buffer A and used to immunize rats.

[00213] Animal immunizations and determination of anti-H5 antibody production by western analysis: Rats were immunized with either H5ΔTMD^{PrePro} or H5ΔTMD^{ProGrl1} mucus based vaccine and then with a booster shot approximately 4 weeks later. To determine production of anti-H5 antibodies, insect derived H5N1 hemagglutinin was resolved by SDS-PAGE and transferred to nitrocellulose. Blots were probed with sera

collected from each rat and then with anti-rat HRP conjugated secondary antibody. Control blots were probed with either pre-immune sera or secondary antibody alone.

[00214] Microneutralization assays: Assays were carried out with the A/Vietnam/1203/2004xPR8 (VN04) strain with a tissue culture infectious $dose_{50}$ ($TCID_{50}$) of 3.2 x 10^8 virus particles/ml. A 1.6 x 10^5 viral particle dose was pre-incubated with serial dilutions of each lot of sera and MDCK cells were then added to the sera/virus mixtures and incubated for 20 hours. Cells were then fixed and the presence of Influenza A virus NP in infected cells was detected by ELISA. The absence of infectivity constitutes a positive neutralization reaction and indicates the presence of virus-specific antibodies in the sera.

[00215] Results. Expression of $H5\Delta TMD^{PrePro}$ or $H5\Delta TMD^{ProGrl1}$ in Tetrahymena was examined by Western analysis of both whole cell lysates and harvested mucus as described herein. Figure 17 shows that each fusion gene was expressed and resolved at a molecular weight >148 kDa indicating the formation of higher order structures of the fusion protein. Such higher order structures are likely a consequence of the H5ΔTMD fusion partner since H5N1 hemagglutinin is known to trimerize. Both $H5\Delta TMD^{PrePro}$ and $H5\Delta TMD^{ProGrl1}$ are recovered in mucus following induced release of mucocyst contents (Figure 17). Sera collected from rats immunized with either mucus-based $H5\Delta TMD^{PrePro}$ or $H5\Delta TMD^{ProGrl1}$ contained anti-hemagglutinin antibodies as judged by detection of insect cell derived recombinant H5N1 hemagglutinin by Western analysis (Figure 3). Detection was specific for sera derived from immunized animals as pre-immune sera or secondary antibody alone failed to detect hemagglutinin (Figure 18). Microneutralization assays confirmed the presence of neutralizing antibodies in sera derived from animals immunized with $H5\Delta TMD^{PrePro}$ or $H5\Delta TMD^{ProGrl1}$ with titers of 10240 and 2560, respectively. As a gauge of efficacy, neutralizing titers of approximately 10,000 are achieved when animals are hyper-immunized with live virus indicating that a similar efficacy is achieved using the mucus-based sub-unit vaccine.

References

Andrews (2000), "Regulated secretion of conventional lysosomes," *Trends Cell Biol.* 10:316-21.

Becker and Rusing (2003), "Structure of N-glycosidic carbohydrates of secretory proteins of *Tetrahymena thermophila*," *J. Eukaryot. Microbiol.* 50:235-239.

Berg *et al.* (1991), "Bispecific antibodies that mediate killing of cells infected with human immunodeficiency virus of any strain," *Proc. Natl. Acad. Sci. USA* 88:4723-4727.

Blomberg *et al.* (1997), "Regulatory sequences for the amplification and replication of the ribosomal DNA minichromosome in *Tetrahymena thermophila*," *Mol. Cell. Biol.* 12:7237-747.

Boldrin *et al.* (2006), "Metallothionein gene from *Tetrahymena thermophila* with a copper-inducible-repressible promoter," *Eukaryot Cell* 2:422-425.

Bowman *et al.* (2005a), "Genomic and proteomic evidence for a second family of dense core granule cargo proteins in *Tetrahymena thermophila*," *J. Eukaryot. Microbiol.* 52:291-7.

Bowman *et al.* (2005b), "Core formation and the acquisition of fusion competence are linked during secretory granule maturation in *Tetrahymena*," *Traffic* 6:303-23.

Bradshaw *et al.* (2003), "Proprotein processing within secretory dense core granules of *Tetrahymena thermophila*," *J. Biol. Chem.* 278:4087-4095.

Bruns and Cassidy-Hanley (2000), "Biolistic transformation of macro-and micronuclei," *Meth. Cell Biol.* 62:501-512.

Bruns *et al.* (1985), "A drug-resistant mutation in the ribosomal DNA of *Tetrahymena*," *Proc. Natl. Acad. Sci. USA* 9:2844-286.

Burgess and Kelly (1987), "Constitutive and Regulated Secretion of Proteins," *Annu. Rev. Cell Biol.* 3:243-293.

Cassidy-Hanley *et al.* (1997), "Germline and somatic transformation of mating *Tetrahymena thermophila* by particle bombardment," *Genetics* 146:135-147.

Chanat *et al.* (1993), "Reduction of the disulfide bond of chromogranin B (secretogranin I) in the trans-Golgi network causes its missorting to the constitutive secretory pathways," *EMBO J.* 12:2159-2168.

Chaudhary *et al.* (1990), "A rapid method of cloning functional variable-region antibody genes in *Escherichia coli* as single-chain immunotoxins," *Proc. Natl. Acad. Sci. USA* 87:1066-1070.

Chen *et al.* (1995), "Regulated secretion of prolactin by the mouse insulinoma cell line beta TC-3," *Biotechnology* 13:1191-7.

Chilcoat *et al.* (1996), "Granule lattice protein 1 (Grl1p), an acidic, calcium-binding protein in *Tetrahymena thermophila* dense-core secretory granules, influences granule size, shape, content organization, and release but not protein sorting or condensation," *J. Cell Biol.* 135(6 Pt 2):1775-87.

Clark *et al.* (2001), "The I-antigens of Ichthyophthirius multifiliis are GPI-anchored proteins," *J. Eukarot. Microbiol.* 48:332-337.

Collins and Wilhelm (1981), "Post-translational cleavage of mucocyst precursors in *Tetrahymena*," *J. Biol. Chem.* 256:10475-10484.

Cool *et al.* (1995), "Identification of the sorting signal motif within pro-opiomelanocortin for the regulated secretory pathway," *J. Biol. Chem.* 270:8723-8729.

Cool *et al.* (1997), "Carboxypeptidase E is a regulated secretory pathway sorting receptor: genetic obliteration leads to endocrine disorders in Cpe(fat) mice," *Cell* 88:73-83.

Cowan *et al.* (2005), "Genetic, genomic, and functional analysis of the granule lattice proteins in *Tetrahymena* secretory granules," *Mol. Biol. Cell.* 16:4046-4060.

de Coninck *et al.* (2000), "Industrial media and fermentation processes for improved growth and protease production by *Tetrahymena thermophila* BIII," *J. Industr. Microbiol. Biotechnol.* 24:285.

Eisen *et al.* (2006), "Macronuclear genome sequence of the ciliate *Tetrahymena thermophila*, a model eukaryote," *PLoS Biol.* 4(9):e286.

Frankel (2000), "The cell biology of *Tetrahymena thermophila,*" *Meth. Cell Biol.* 62:27-125.

Gaertig and Gorovsky (1992), "Efficient mass transformation of *Tetrahymena thermophila* by electroporation of conjugants," *Proc. Natl. Acad. Sci. USA* 89:9196-9200.

Gaertig *et al.* (1994), "High frequency vector-mediated transformation and gene replacement in *Tetrahymena*," *Nucleic Acids Res.* 22:5391-5398.

Gaertig *et al.* (1999), "Surface display of a parasite antigen in the ciliate *Tetrahymena thermophila*," *Nature Biotech.* 17:462-465.

Gaertig and Kapler (2000), "Transient and stable DNA transformation of *Tetrahymena thermophila* by electroporation," *Meth. Cell Biol.* 62:486-500.

Glombik *et al.* (1999), "The disulfide-bonded loop of chromogranin B mediates membrane binding and directs sorting from the trans-Golgi network to secretory granules," *EMBO J.* 18:1059-1070.

Grampp *et al.* (1992), "Use of regulated secretion in protein production from animal cells: an overview," *Adv Biochem Eng Biotechnol.* 46:35-62.

Gundelfinger *et al.* (2003), "Temporal and spatial coordination of exocytosis and endocytosis," *Nat. Rev. Mol. Cell Biol.* 4(2):127-39.

Haddad *et al.* (2002), "New class of cargo protein in *Tetrahymena thermophila* dense core secretory granules," *Eukaryot. Cell.* 1:583-93.

Hai *et al.* (2000), "Knockout heterokaryons enable facile mutagenic analysis of essential genes in *Tetrahymena*," *Meth. Cell Biol.* 62:513-531.

Hellenbroich *et al.* (1999), "Cultivation of *Tetrahymena thermophil*a in a 1.5 m3 airlift bioreactor," *Appl. Microbiol. Biotechnol.* 51:447.

Huse *et al.* (1989), "Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda," *Science* 246:1275-1281.

Langer (2005), 3rd Annual Report and Survey of Biopharmaceutical Manufacturing, Capacity and Production,. BioPlan Associates, Inc.

Larsen *et al.* (1999), "Cloning and characterization of the gene encoding the highly expressed ribosomal protein l3 of the ciliated protozoan *Tetrahymena thermophila*. Evidence for differential codon usage in highly expressed genes," *Cell Biol. Int.* 23(8):551-60.

Lin *et al.* (2002), "The use of synthetic genes for the expression of ciliate proteins in heterologous systems," *Gene* 288(1-2):85-94.

Madeddu *et al.* (1994), "Protein processing and morphogenesis of secretory granules in *Paramecium*," *Biochimie.* 76:329-35.

Maihle and Satir (1986), "Protein secretion in *Tetrahymena thermophila*: Characterization of the major proteinaceous secretory proteins," *J. Biol. Chem.* 261(16):7566-70.

McCloskey (2004), "Creating therapeutic proteins from bioengineered systems," *Biotechnology Healthcare* (September issue) p. 58.

Melia *et al.* (1998), "Mutational analysis of regulated exocytosis in *Tetrahymena*," *J. Cell Sci.* 111(Pt. 1):131-40.

Miller and Moore (1990), "Regulated secretion," *Curr. Opin. Cell Biol.* 2:642-647.

Mullinax *et al.* (1990), "Identification of human antibody fragment clones specific for tetanus toxoid in a bacteriophage lambda immunoexpression library," *Proc. Natl. Acad. Sci. USA* 87:8095-8099.

Orias *et al.* (1988), "Replacement of the macronuclear ribosomal RNA genes of a mutant *Tetrahymena* using electroporation," *Gene* 2:295-301.

Pan et al. (1982), "Allele-specific, selective amplification of a ribosomal RNA gene in *Tetrahymena thermophila*," Cell 3:595-604.

Pavlou and Reichert (2004), "Recombinant protein therapeutics--success rates, market trends and values to 2010," *Nat. Biotechnol.* 22:1513-1519.

Pluckthun (1990), "Antibodies from *Escherichia coli*," *Nature* 347:497-498.

Roy *et al.* (1991), "Investigation of a possible role of the amino-terminal pro-region of proopiomelanocortin in its processing and targeting to secretory granules," *Mol. Cell. Endocrinol.* 82:237-250.

Satir (1977), "Dibucaine-induced synchronous mucocyst secretion in *Tetrahymena*," *Cell Biol. Int. Rep.* 1:69-73.

Shang *et al.* (2002), "A robust inducible-repressible promoter greatly facilitates gene knockouts, conditional expression, and overexpression of homologous and heterologous genes in *Tetrahymena thermophila*," *Proc. Natl. Acad. Sci. USA* 6:3734-379.

Spangler and Blackburn (1985), "The nucleotide sequence of the 17S ribosomal RNA gene of *Tetrahymena thermophila* and the identification of point mutations resulting in resistance to the antibiotics paromomycin and hygromycin," *J. Biol. Chem.* 10:6334-6340.

Taniguchi *et al.* (1985), "Carbohydrates of lysosomal enzymes secreted by *Tetrahymena pyriformis*," *J. Biol. Chem.* 260:13941-13946.

Tondravi and Yao (1986), "Transformation of *Tetrahymena thermophila* by microinjection of ribosomal RNA genes," *Proc. Natl. Acad. Sci USA* 83:4369-73.

Turkewitz (2004), "Out with a bang! *Tetrahymena* as a model system to study secretory granule biogenesis," *Traffic* 5:63-8.

Turkewitz *et al.* (2000), "Regulated protein secretion in *Tetrahymena thermophila*," *Meth. Cell Biol.* 62:347-362.

Turkewitz *et al.* (2002), "Functional genomics: the coming of age for *Tetrahymena thermophila*," *TRENDS in Genetics* 18:35-40.

Vayssié *et al.* (2000), "Molecular genetics of regulated secretion in *Paramecium*," *Biochimie.* 82:269-88.

Weide *et al.* (2006), "Secretion of functional human enzymes by *Tetrahymena thermophila*," *BMC Biotechnol.* 6:19.

Williams *et al.* (1980), "Studies of membrane formation in *Tetrahymena*: Identification of membrane proteins and turnover rates in non-growing cells," *J. Biol. Chem.* 255:296-303.

Wood *et al.* (1990), "High level synthesis of immunoglobulins in Chinese hamster ovary cells," *J. Immunol.* 145:3011-3016.

Wuitschick and Karrer (2000), "Codon usage in *Tetrahymena thermophila*," *Meth. Cell Biol.* 62:565-8.

Wuitschick and Karrer (1999), "Analysis of genomic G + C content, codon usage, initiator codon context and translation termination sites in *Tetrahymena thermophila*," *J. Eukaryot. Microbiol.* 46:239–247.

Yang and Hsieh (2001), "Regulated secretion of proinsulin/insulin from human hepatoma cells transduced by recombinant adeno-associated virus," *Biotechnol. Appl. Biochem.* 33:133–140.

Yu and Blackburn (1989), "Transformation of *Tetrahymena thermophila* with a mutated circular ribosomal DNA plasmid vector," *Proc. Natl. Acad. Sci. USA* 21:8487-891.

Yu *et al.* (1988), "Circular ribosomal DNA plasmids transform *Tetrahymena thermophila* by homologous recombination with endogenous macronuclear ribosomal DNA," *Proc. Natl. Acad. Sci. USA* 14:5151-5155

Zhang *et al.* (1999), "Identification of a novel prohormone sorting signal-binding site on carboxypeptidase E, a regulated secretory pathway-sorting receptor," *Mol. Endocrinol.* 13:527-536.

WHAT IS CLAIMED IS:

1. A method for production of a heterologous soluble polypeptide by a ciliate, the method comprising:

(a) transforming said ciliate with a nucleic acid encoding a fusion protein comprising from N-terminus to C-terminus:

(i) a mucocyst-targeting polypeptide of a mucocyst-targeted protein which is cleaved by a protease endogenous to said mucocyst; and

(ii) a heterologous polypeptide;

wherein expression of said fusion protein results in trafficking of said fusion protein to mucocysts within said ciliate and cleavage of said mucocyst-targeting polypeptide to release said heterologous soluble polypeptide within said mucocysts;

(b) stimulating regulated secretion from said mucocysts of said ciliate, whereby an extracellular matrix is formed; and

(c) separating said heterologous soluble polypeptide from said extracellular matrix and said ciliates.

2. A method for production of a heterologous soluble polypeptide by a ciliate, the method comprising:

(a) transforming said ciliate with a nucleic acid encoding a first fusion protein comprising from N-terminus to C-terminus:

(i) a heterologous polypeptide; and

(ii) at least a mucocyst-targeting polypeptide of a mucocyst-targeted protein;

wherein expression of said first fusion protein results in trafficking of said first fusion protein to mucocysts within said ciliate; and

wherein an endogenous protease within said mucocysts cleaves a cleavage site within said mucocyst-targeting polypeptide and removes any sequences C-terminal to said cleavage site, thereby producing a second fusion protein within said mucocysts;

(b) stimulating regulated secretion from said mucocysts of said ciliate, whereby an extracellular matrix is formed; and

(c) separating said heterologous polypeptide from said extracellular matrix and said ciliates.

3. A method for producing a desired heterologous polypeptide in a culture of ciliates, the method comprising:

(a) expressing a fusion protein comprising said heterologous polypeptide and a polypeptide comprising at least one mucocyst-targeting polypeptide of a mucocyst-targeted protein in said ciliates;

(b) stimulating regulated secretion from mucocysts of said ciliates, whereby an extracellular matrix is formed by said secretion;

(c) separating said extracellular matrix from said ciliates; and

(d) isolating said fusion protein from said extracellular matrix.

4. The method of any of claim 1-3 wherein said mucocyst-targeted protein is a Grl protein.

5. The method of any of claim 1-3 wherein said mucocyst-targeted protein is selected from the group consisting of a Grl-1 protein, a Grl-2 protein a Grl-3 protein, a Grl-4 protein, a Grl-5 protein, a Grl-6 protein, a Grl-7 protein, a Grl-8 protein, a Grl-9 protein, and a Grl-10 protein.

6. The method of any of claim 1-3 wherein said mucocyst-targeted protein is an Igr protein or a granule tip protein.

7. The method of any of claim 1-3 wherein said mucocyst-targeted protein is a β/γ crystalline domain or a C-terminal crystallin fold containing protein.

8. The method of claim 5 wherein said mucocyst-targeting domain comprises a pro-domain of said Grl protein.

9. The method of any of claim 1-3 wherein said fusion protein further comprises an endoplasmic reticulum-targeting polypeptide N-terminal to said mucocyst-targeting polypeptide.

10. The method of claim 9 wherein said endoplasmic reticulum-targeting polypeptide is a pre-domain of a Grl protein.

11. The method of claim 9 wherein said endoplasmic reticulum-targeting polypeptide is heterologous to said mucocyst-targeting polypeptide.

12. The method of claim 2 wherein said first fusion protein further comprises a second protease cleavage site between said heterologous polypeptide and said mucocyst-targeting polypeptide.

13. The method of claim 12, further comprising the step of reacting said second fusion protein with a second protease which cleaves said second protease cleavage site after step (b).

14. The method of claim 12, further comprising the step of reacting said second fusion protein with a second protease which cleaves said second protease cleavage site after step (c).

15. A method for production of a heterologous soluble polypeptide by a ciliate, the method comprising:

(a) transforming said ciliate with a nucleic acid encoding a fusion protein comprising from N-terminus to C-terminus:
    (i) a soluble polypeptide endogenous to said mucocyst;
    (ii) a protease cleavage site; and
    (iii) a heterologous polypeptide;
wherein expression of said fusion protein results in trafficking of said fusion protein to mucocysts within said ciliate;
(b) stimulating regulated secretion from said mucocysts of said ciliate, whereby an extracellular matrix is formed by said secretion;
(c) separating said fusion protein from said extracellular matrix and said ciliates; and
(d) obtaining said heterologous soluble polypeptide from said fusion protein.

16. The method of claim 15 wherein said mucocyst-targeted protein is a β/γ crystalline domain containing or a C-terminal crystallin fold protein.

17. The method of claim 15 wherein step (d) comprises the step of reacting said heterologous soluble polypeptide with a protease which cleaves said protease cleavage site.

18. The method of any one of claims 15-17 wherein said fusion protein further comprises an endoplasmic reticulum-targeting polypeptide N-terminal to said heterologous polypeptide.

19. The method of claim 18 wherein expression of said fusion protein results in trafficking of said fusion protein to mucocysts within said ciliate and cleavage of said endoplasmic reticulum-targeting polypeptide.

20. The method of claim 18 wherein said endoplasmic reticulum-targeting polypeptide is a pre-domain of a Grl protein.

21. The method of claim 18 wherein said endoplasmic reticulum-targeting polypeptide is heterologous to said soluble polypeptide endogenous to said mucocyst.

22. A protein preparation produced by a method of any one of claims 1-21.

23. A nucleic acid having a sequence encoding the fusion protein of any one of claims 1-21.

24. A transgenic ciliate comprising nucleic acid of claim 23.

25. A nucleic acid having a sequence encoding a fusion protein comprising:
    (a) at least one mucocyst-targeting polypeptide;
    (b) a heterologous polypeptide; and
    (c) a cleavable linker between said mucocyst-targeting polypeptide and said heterologous polypeptide.

26. A transgenic ciliate comprising:
    a nucleic acid having a sequence encoding:
        (a) at least one mucocyst-targeting polypeptide;
        (b) a heterologous polypeptide; and
        (c) a cleavable linker between said mucocyst-targeting polypeptide and said heterologous polypeptide.

27. A protein preparation comprising:
    (a) an extracellular matrix formed by regulated secretion by ciliates; and
    (b) a fusion protein encoded by said ciliates.

28. A vaccine preparation comprising:

(a) an extracellular matrix formed by regulated secretion by ciliates; and

(b) a fusion protein encoded by said ciliates;

wherein said fusion protein comprises an immunogenic polypeptide.

29. A vaccine preparation comprising:

(a) an extracellular matrix formed by regulated secretion by ciliates; and

(b) at least two different fusion proteins encoded by said ciliates;

wherein said fusion proteins comprise different immunogenic polypeptides derived from the same pathogen or tumor cell.

30. A vaccine preparation comprising:

(a) an extracellular matrix formed by regulated secretion by ciliates; and

(b) at least two different fusion proteins encoded by said ciliates;

wherein said fusion proteins comprise different immunogenic polypeptides derived from different pathogens and/or tumor cells.

31. A vaccine preparation comprising:

(a) an extracellular matrix formed by regulated secretion by ciliates; and

(b) at least two different fusion proteins encoded by said ciliates;

wherein one of said fusion proteins comprises an immunogenic polypeptide derived from a pathogen and/or tumor cell, and one of said fusion proteins comprises an immunostimulatory polypeptide or a receptor that binds an immunostimulatory polypeptide.

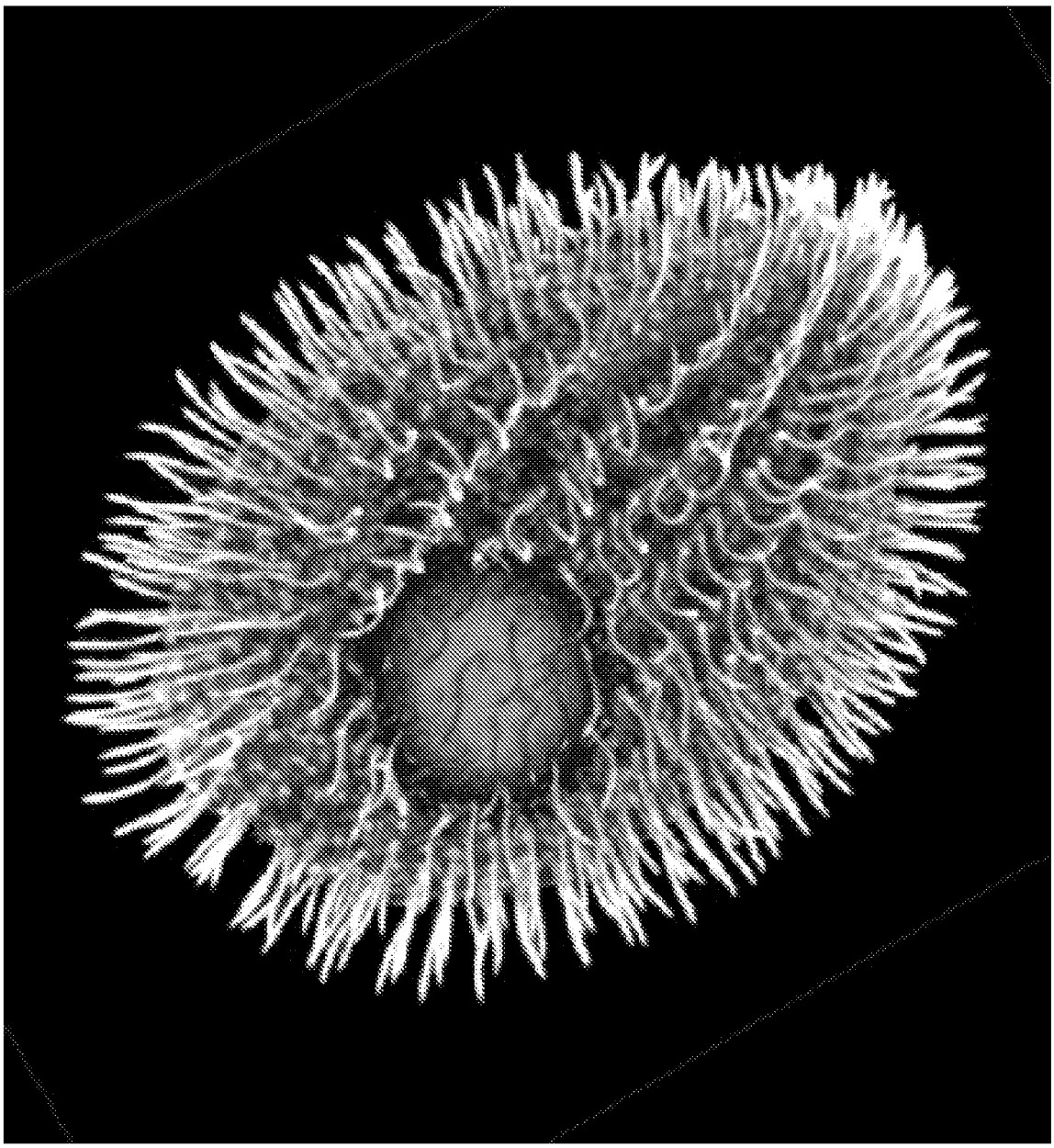


Figure 1

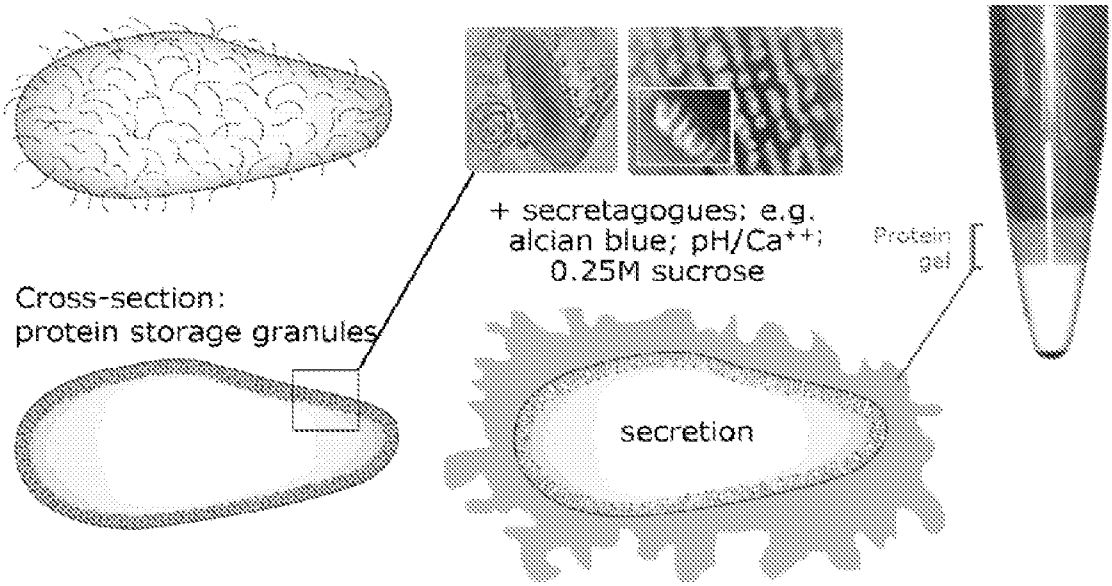

+ secretagogues; e.g.
alcian blue; pH/Ca++;
0.25M sucrose
Protein
gel
Cross-section:
protein storage granules
secretion

Figure 2

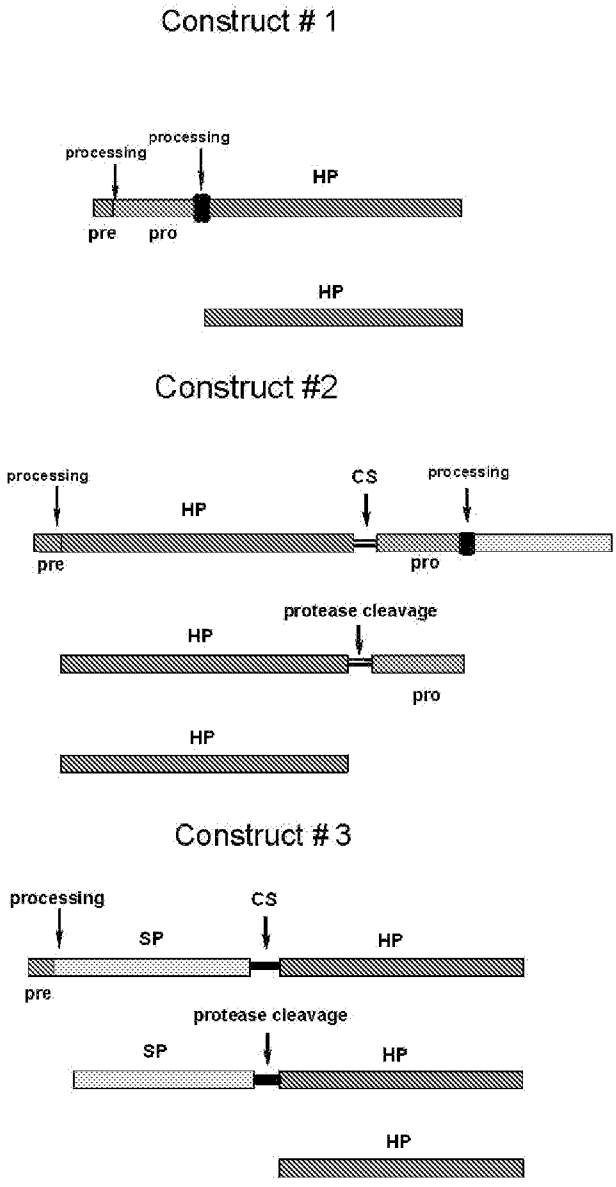
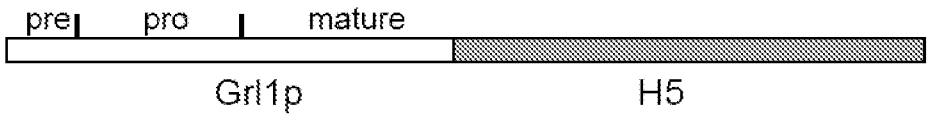

Construct # 1
processing
processing
HP
pre
pro
HP
Construct #2
processing
CS
processing
HP
pre
pro
protease cleavage
HP
pro
HP
Construct # 3
processing
CS
SP
HP
pre
protease cleavage
SP
HP
HP

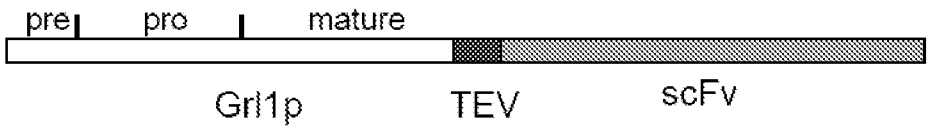
Figure 3

A



MGSKLLVVLFGFLALAAATNQSEEEGSYTIDQAANLLNDLLADSQQNLSDLQAAWANKEPLLQGVIAGLES
DLANKQAECADLQGTLDADQASLDEAEAYVAWLQDRIAANHKQIDDLLNRRCQQNGNYIEGLKNDKLALAL
LQFLEAQIQNKESFSFLQKKNFMKKLTRFLSIYKTGNYQQLALLEKEYVNADDYSVNPDYSTGDRTADEIG
SGHIDNDKGDIDVADFQEGEPKGWYQVKQELLDLLHNLEQTIEAKIQQAQEDEVNSNSAAADFKSKLEHEI
QVYEPELAKWQQTVAALTATVAQDHENVNNCHSQEAAIQANLDAANQDYANEKATFEHKQANLQEEIEIFI
EVIAYYDDNVQNAGEDLKERVEDYSDGNFDDAATYENRQVPNIDFINENLYFQGHHHHHHCYPYDVPDYASLDIQMTQSPSSLSASVGDR
VTITCRASQDIRNYLNWYQQKPGKAPKLLIYYTSRLLPGVPSRFSGSGSGTDYTLTISSLQEEDIATYYCQQGN
TLPWTFGQGTKVEIKRTGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAASGYAFSSSWMNWV
RQAPGKGLEWVGRIYPGLGDTNYNGKFKGRATISADKSSSTAYLQMNSLRAEDTAVYYCARPSGLLFYAMDYWGQ
GTLVTVSS

B



MSKLLVVLFGFLALAAATNQSEEEGSYTIDQAANLLNDLLADSQQNLSDLQAAWANKEPLLQGVIAGLESDLAN
KQAECADLQGTLDADQASLDEAEAYVAWLQDRIAANHKQIDDLLNRRCQQNGNYIEGLKNDKLALALLQFLEAQ
IQNKESFSFLQKKNFMKKLTRFLSIYKTGNYQQLALLEKEYVNADDYSVNPDYSTGDRTADEIGSGHIDNDKGD
IDVADFQEGEPKGWYQVKQELLDLLHNLEQTIEAKIQQAQEDEVNSNSAAADFKSKLEHEIQVYEPELAKWQQT
VAALTATVAQDHENVNNCHSQEAAIQANLDAANQDYANEKATFEHKQANLQEEIEIFIEVIAYYDDNVQNAGED
LKERVEDYSDGNFDDAATYENRQVPNIDFINENLYFQGHHHHHHCYPYDVPDYASLDIQMTQSPSSLSASVGDR
VTITCRASQDIRNYLNWYQQKPGKAPKLLIYYTSRLLPGVPSRFSGSGSGTDYTLTISSLQEEDIATYYCQQGN
TLPWTFGQGTKVEIKRTGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAASGYAFSSSWMNWV
RQAPGKGLEWVGRIYPGLGDTNYNGKFKGRATISADKSSSTAYLQMNSLRAEDTAVYYCARPSGLLFYAMDYWGQ
GTLVTVSS

Figure 4

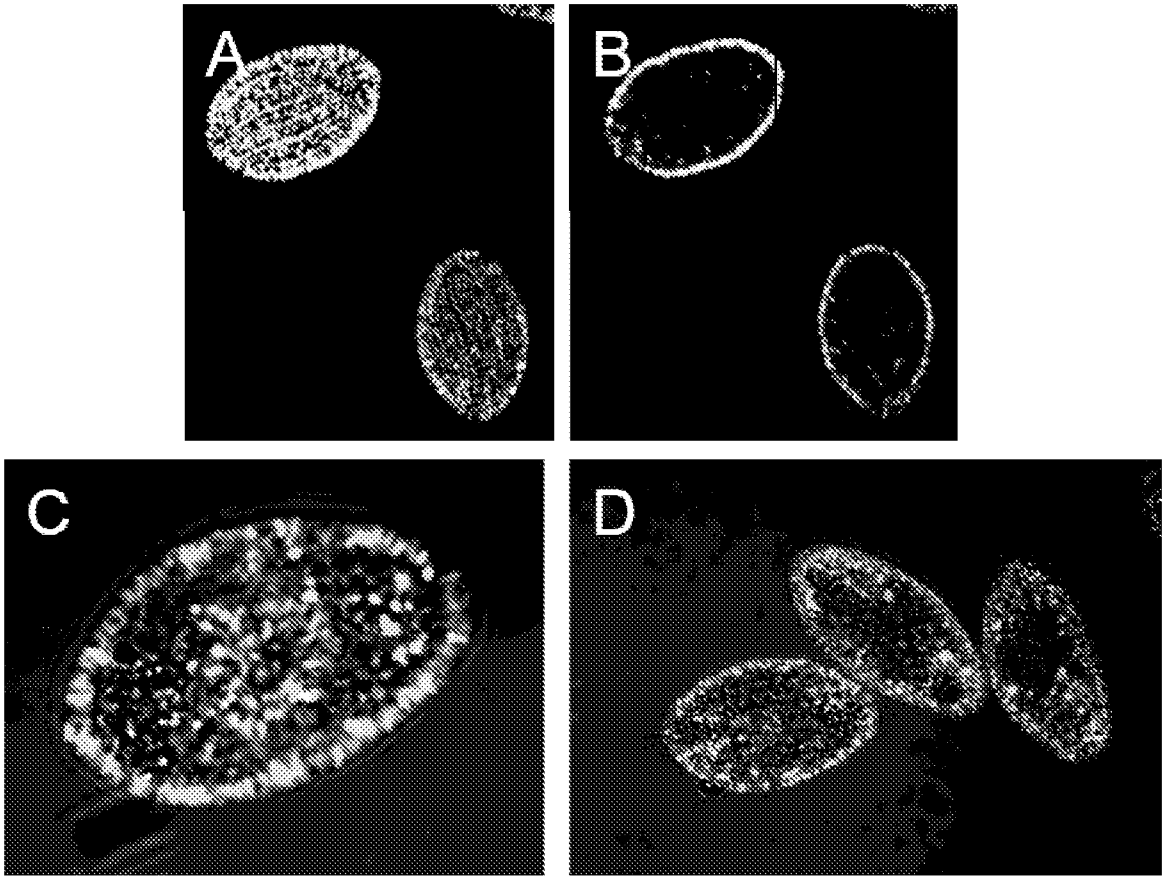


Figure 5

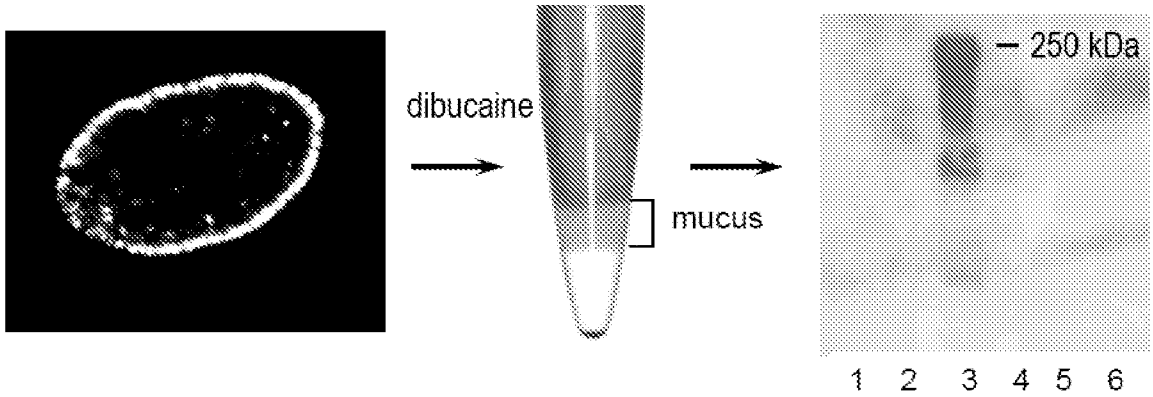
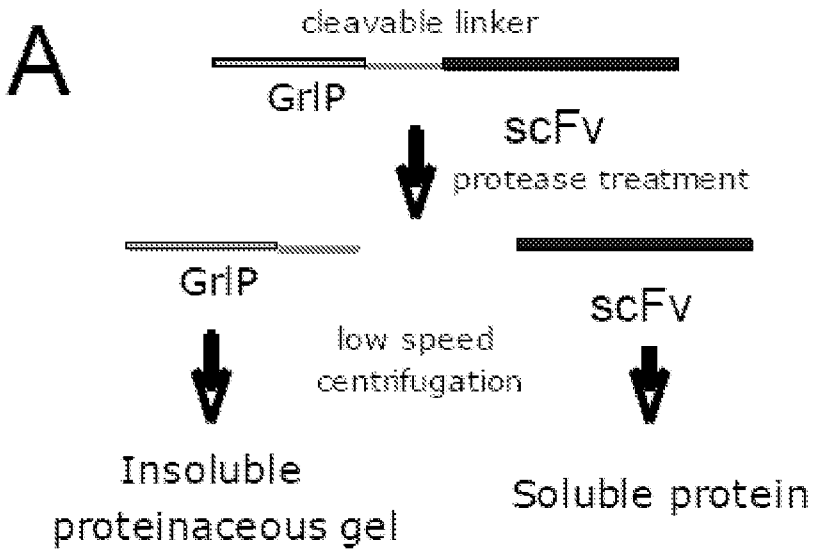


Figure 6


A
cleavable linker
GrIP
scFv
protease treatment
GrIP
scFv
low speed
centrifugation
Insoluble
proteinaceous gel
Soluble protein

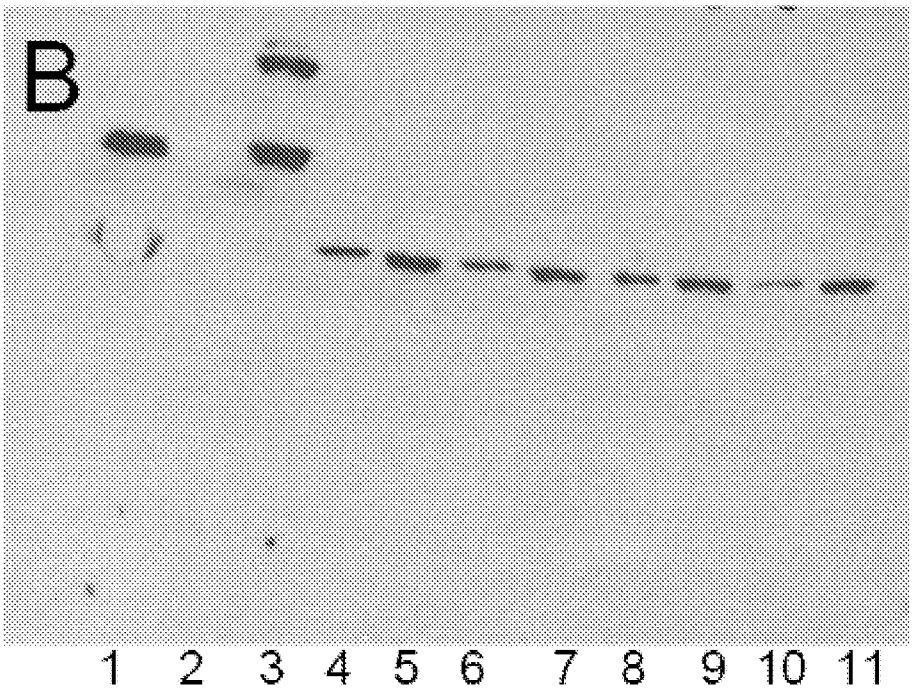
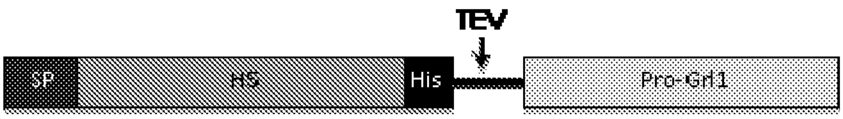

B
1 2 3 4 5 6 7 8 9 10 11

Figure 7

A

H5ΔTMD[ProGrl1]



<u>**MGSEKIVLLFAIVSLVKS**</u>DQICIGYHANNSTEQVDTIMEKNVTVTHAQDILEKTHNGKLCDLD
GVKPLILRDCSVAGWLLGNPMCDEFINVPEWSYIVEKANPVNDLCYPGDFNDYEELKHLLSRIN
HFEKIQIIPKSSWSSHEASLGVSSACPYQGKSSFFRNVVWLIKKNSTYPTIKRSYNNTNQEDLLVL
WGIHHPNDAAEQTKLYQNPTTYISVGTSTLNQRLVPRIATRSKVNGQSGRMEFFWTILKPNDAI
NFESNGNFIAPEYAYKIVKKGDSTIMKSELEYGNCNTKCQTPMGAINSSMPFHNIHPLTIGECPK
YVKSNRLVLATGLRNSPQRERRRKKRGLFGAIAGFIEGGWQGMVDGWYGYHHSNEQGSGYA
ADKESTQKAIDGVTNKVNSIIDKMNTQFEAVGREFNNLERRIENLNKKMEDGFLDVWTYNAEL
LVLMENERTLDFHDSNVKNLYDKVRLQLRDNAKELGNGCFEFYHKCDNECMESVRNGTYDY
PQYSEEARLKREEIS<u>***HHHHHHHHHH***</u><u>ENLYFQG</u> ***TWQSEEEGSVTIDQAANLLEDLLADSQQWIS
DLQAAVANKEPLLQGVIAGLESDLANWQAECADLQGTLDADQASLDEAKAVVAWLQDNIAAN
HKQIDNLLNERCQQWGNVIEGLENDNLALALLQFLEAQIQNEESPSPLQEEIFNEELTEFLSY
KTGNYQQLALLEEKYVEA***DDYSVMPDYSTGDRTADEIGSGHIDNDKGDIDVADFQEGERKG
WYQVKQELLDLLHNLEQTIEAKIQQAQEDEVNSNSAAADFKSKLEHEIQVVERELAKWQ
QTVAALTATVAQDHENWVNNCHSQEAAIQANLDAANQDYANEKATFEHKQANLQEEIKIEI
EVIAYYDDNVQNAGEDLKERVEDYSDGNFDDAATYENRQVPNIDFIN

<u>**Bold Underlined:**</u> <u>**H5N1 HA Signal peptide**</u>

Black Text: H5N1 HA ΔTMD

<u>***Underlined Bold Italicized text***</u>: **10 x His tag**

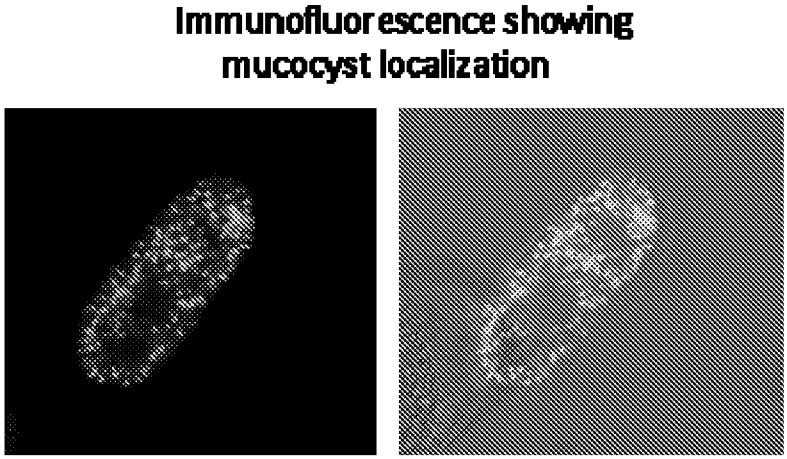
<u>**Undelined Text**</u>: **TEV Protease cleavage site**

Bold Italics Text: Grl1 Pro-domain

Bold Text: Mature Grl1

Figure 8A

B



C

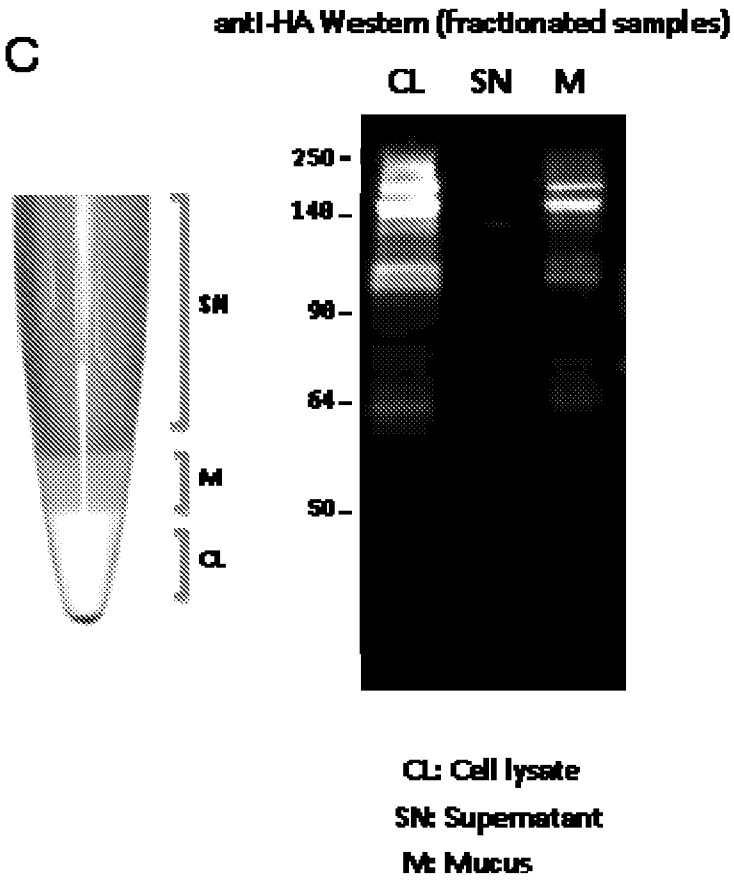


Figure 8B-8C

A

H5ΔTMD[PrePro]

MGSMKKLLVVLFGFLALAAA ***TWQSEEEGSYTIDQAANILLNDLLADSQQW ISDLQAAVANMEKPLLQGVLAGLESDLANMQAECADLQGTLDADQASLDEA EAYVAVLQDRIAANMKQIDDLLNMPRCQQWGWYIEGLKWDKLALALLQFLE AQIQWKESFSFLQKKMFNKKLTMFLSIVKTGWYQQLALLKKMYVNA***DQICIG YHANNSTEQVDTIMEKNVTVTHAQDILEKTHNGKLCDLDGVKPLILRDCS VAGWLLGNPMCDEFINVPEWSYIVEKANPVNDLCYPGDFNDYEELKHLLS RINHFEKIQIIPKSSWSSHEASLGVSSACPYQGKSSFFRNVVWLIKKNSTYPTI KRSYNNTNQEDLLVLWGIHHPNDAAEQTKLYQNPTTYISVGTSTLNQRLVP RIATRSKVNGQSGRMEFFWTILKPNDAINFESNGNFIAPEYAYKIVKKGDST IMKSELEYGNCNTKCQTPMGAINSSMPFHNIHPLTIGECPKYVKSNRLVLAT GLRNSPQRERRRKKRGLFGAIAGFIEGGWQGMVDGWYGYHHSNEQGSGY AADKESTQKAIDGVTNKVNSIIDKMNTQFEAVGREFNNLERRIENLNKKME DGFLDVWTYNAELLVLMENERTLDFHDSNVKNLYDKVRLQLRDNAKELG NGCFEFYHKCDNECMESVRNGTYDYPQYSEEARLKREEIS***HHHHHHHHHH***

Bold Underlined: Grf1 signal (pre) peptide

Bold Italics Text: Grf1 Pro-domain

Black Text: H5N1 HA ΔTMD

Bold underlined italicized Text: 10 x His tag

Figure 9A

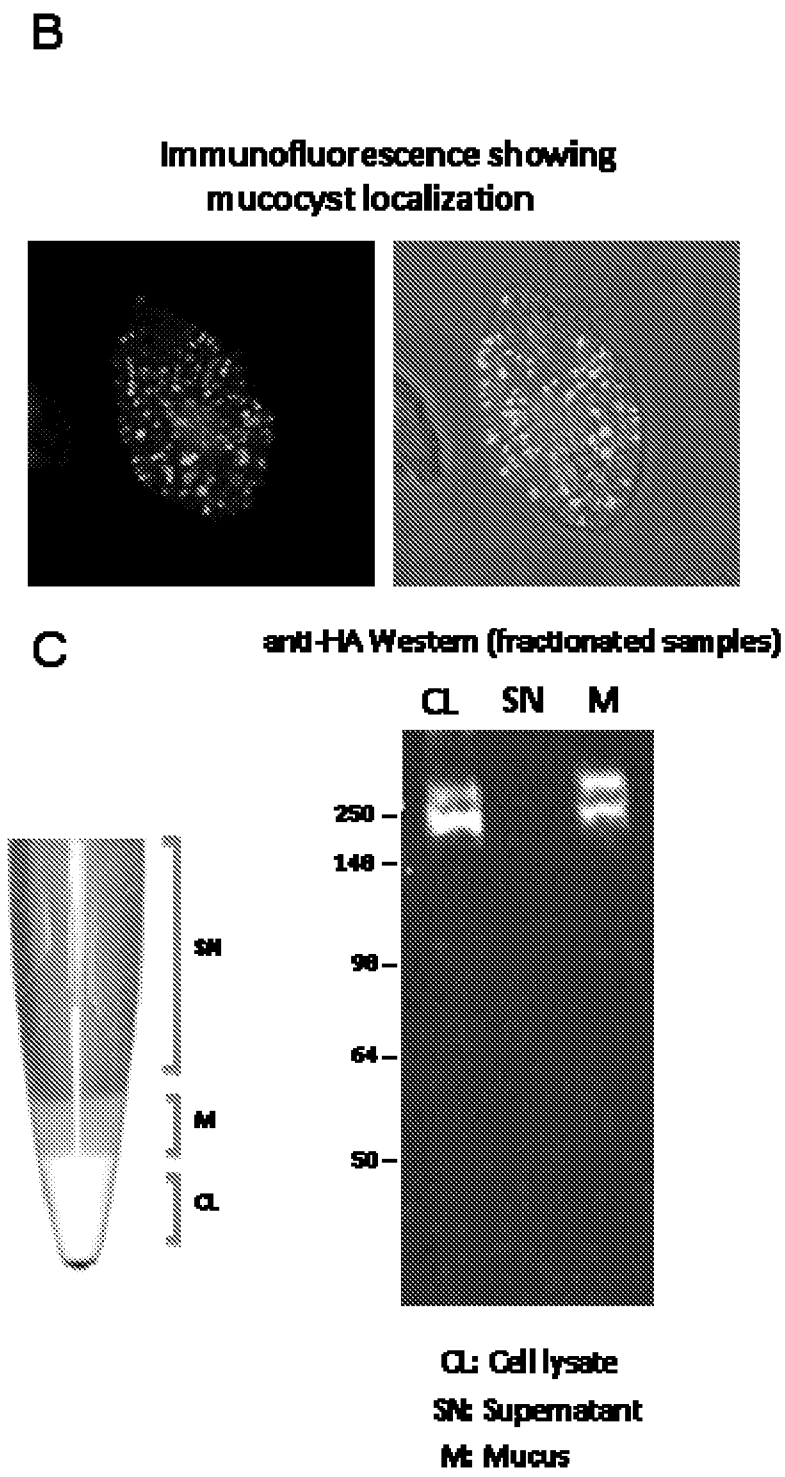

B
Immunofluorescence showing mucocyst localization
C
anti-HA Western (fractionated samples)
CL
SN
M
250
148
98
64
50
SN
M
CL
CL: Cell lysate
SN: Supernatant
M: Mucus

Figure 9B-9C

A

MGSRKIILLLAIISLALCQKLIVEKVAGQYWSGQKFAKSVQNSQVWDYQDFAIY
GVFKIDSSYQIAEWSTGFHFTSWQDKDVTWASAPGDRVLAFWVIGWTLHWPTY
SLARGWTWVYETLSFAAGDTWKVAFIYVTHGSSQQAQYVYYLLPSSGVVTKWI
ASITHKTSTFYQIWVGQSFSFKYFPGSFVRLSLIAGPWAYRESGFEQFQWIQPDV
VPSCPILFTGCWYSGKGDSLCQSSPSYWVTAVHSIYLPAWFTATLHDQAWYAGK
KIVYSQSIECITQLWVAYLLSTHAITIEDETKTVLRRWWRRWENLYFQGDQICIGY
HANWSTEQVDTIWEKNVTVTHAQDILEKTHNGKLCDLDGVKPLILRDCSVAGWLL
GNPMCDEFINVPEWSYIVEKANPVNDLCYPGDFNDYEELKHLLSRINHFEKIQIIPKS
SWSSHEASLGVSSACPYQGKSSFFRNVVWLIKKNSTYPTIKRSYNNTNQEDLLVL
GIHHPNDAAEQTKLYQNPTTYISVGTSTLNQRLVPRIATRSKVNGQSGRMEFFWTIL
KPNDAINFESNGNFIAPEYAYKIVKKGDSTIMKSELEYGNCNTKCQTPMGAINSSMP
FHNIHPLTIGECPKYVKSNRLVLATGLRNSPQRERRRKKRGLFGAIAGFIEGGWQGM
VDGWYGYHHSNEQGSGYAADKESTQKAIDGVTNKVNSIIDKMNTQFEAVGREFN
NLERRIENLNKKMEDGFLDVWTYNAELLVLMENERTLDFHDSNVKNLYDKVRLQ
LRDNAKELGNGCFEFYHKCDNECMESVRNGTYDYPQYSEEARLKREEIS######
####

Bold Underlined: Igr1 Signal peptide

Bold Text: Mature Igr1

Underlined Text: TEV Protease cleavage site

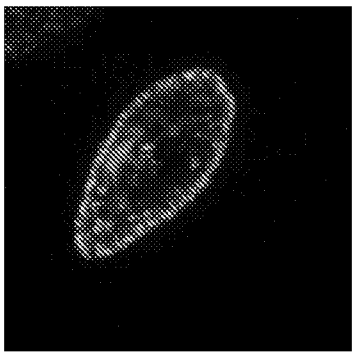
Black Text: H5N1 HA ΔTMD

Italicized Text: 10 x His tag

Figure 10A

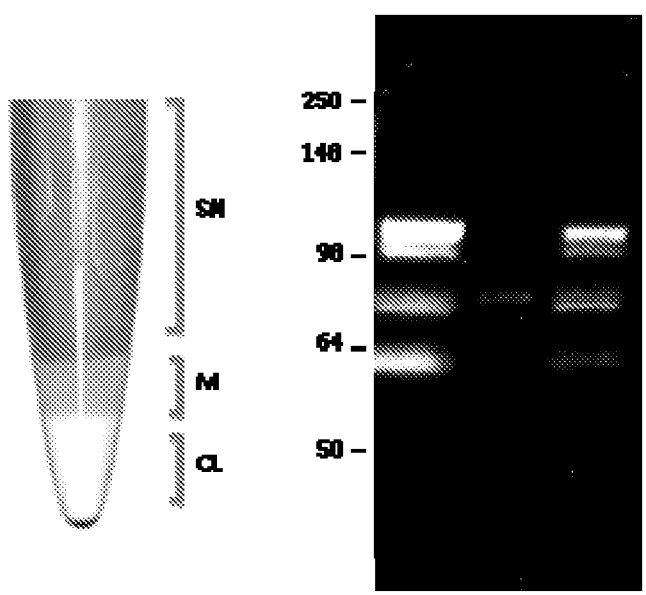
B

Immunofluorescence showing mucocyst localization



C

anti-HA Western (fractionated samples)



CL: Cell lysate
SN: Supernatant
M: Mucus

Figure 10B-10C

A

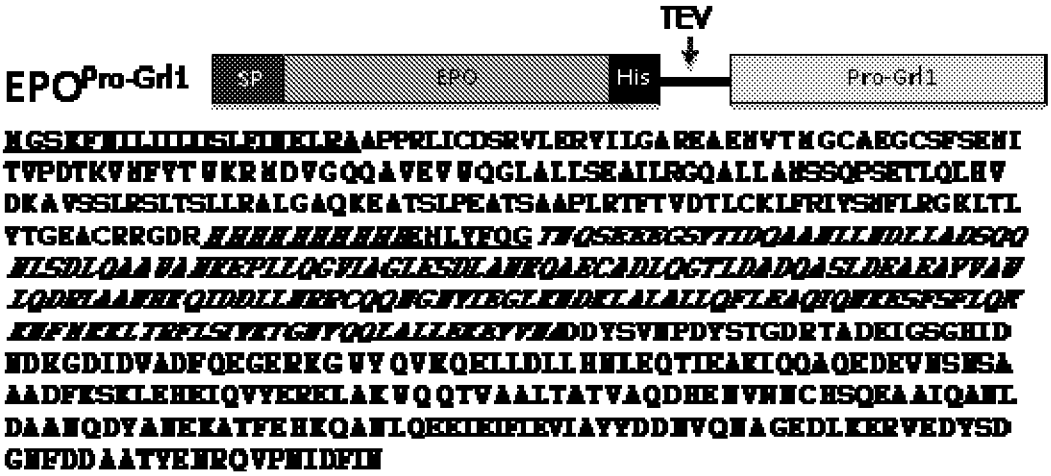


Figure 11A

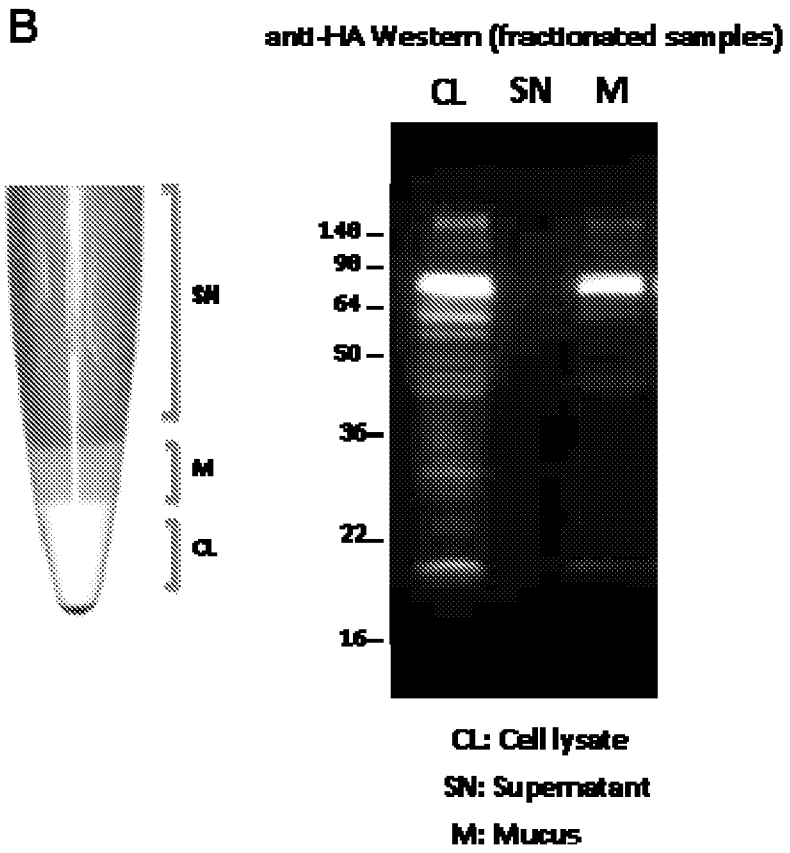
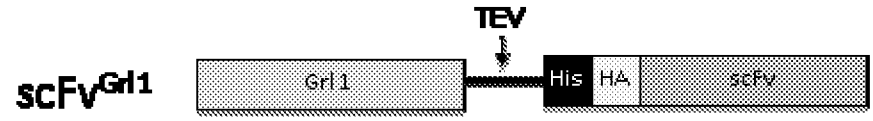

B
anti-HA Western (fractionated samples)
CL
SN
M
140
98
64
50
36
22
16
SN
M
CL
CL: Cell lysate
SN: Supernatant
M: Mucus

Figure 11B

A



MGSMKLLVVLFGFLALAAA ***TWQSEEEGSVTTDQAAPLLMDLLADSQQ***
MTSDLQAAVAMEEPLLQGVTAGLESPLAMRQAECADLQGTLDADQAS
LDEAEAVVAVLQDMLAAMMFQIDDLLMERCQQNGMVIEGLKNDELAI
ALLQFLEAQTQWEESPSFLQEKMFMKKLTPFLSVMTGMVPQQLALLEKE
VFMADDYSVMPDYSTGDRTADEIGSGHIDMDKGDIDVADFQEGERK
GVYQVKQELLDLLHMLEQTIEAKIQQAQEDEVMSMSAAADFKSKL
EMEIQVVEMELAKVQQTVAALTATVAQDHEMVMMCHSQEAAIQA
MLDAAMQDVAMEKATFEMKQAMLQEKIKIFIEVIAYYDDMVQMAGE
DLKKRVEDYSDGMFDDAATYEMRQVPMIDFIM<u>EMLYFQG</u>*HHHHHH*
C*YPYDVPDYA*SLDIQMTQSPSSISASVGDRVTITCRASQDIRNYLNWYQ
QKPGKAPKLLIYYTSRLLPGVPSRFSGSGSGTDYTLTISSQEQEDIATYY
CQQGNTLPWTFGQGTKVEIKRTGGGGSGGGGSGGGGSGGGGSEVQL
VESGGGLVQPGGSLRLSCADSGYAFSSSWMNWVRQAPGKGLEWVGR
IYPGDGDTNYNGKFKGRATISADKSSSTAYLQMNSLRAEDTAVYYCA
RSGLLRYAMDYWGQGTLVTVSS

<u>Bold Underlined: Grl1 Signal (pre) peptide</u>

Bold Italics Text: Grl1 Pro-domain

Bold Text: Mature Grl1

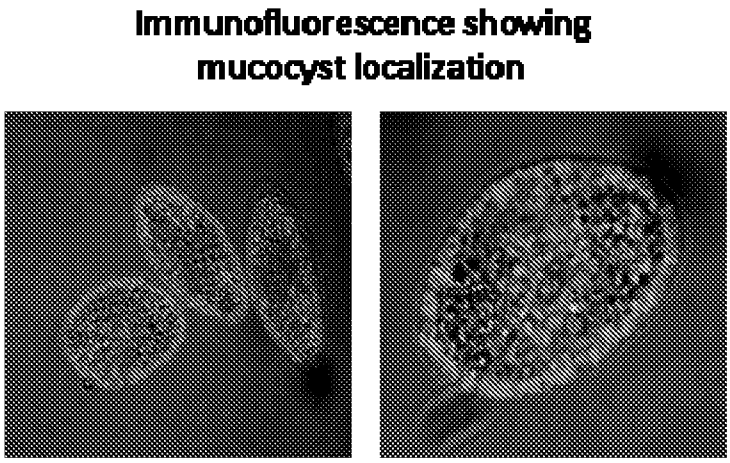
Underlined Text: TEV Protease cleavage site

Bold, Italicized underlined Text: 6 x His tag

Black Text: scFv

Figure 12A

B



C

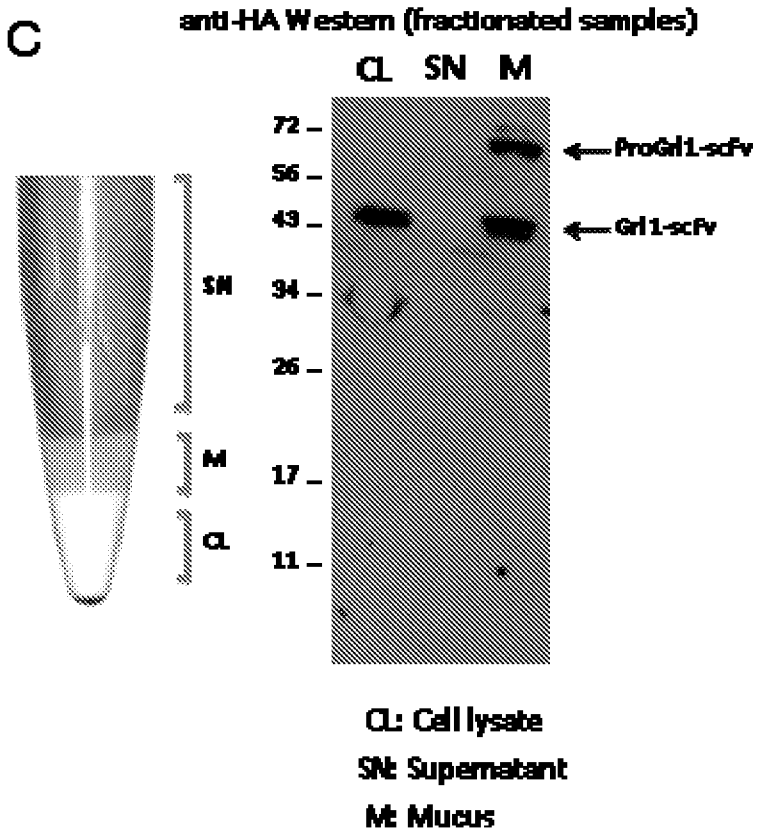
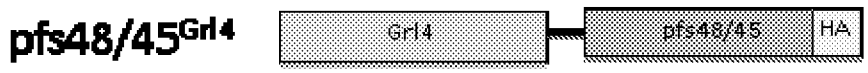


Figure 12B-12C

A



MGSRYAALFLLALISHAVYA *PSLRESSDANETSPALEWLRFTGKESPI*
AEQIESAVELHLTTQGLVIDDVIIL VEQAQKDVAHEVVALQAEYTAKRGA
LKDQIHTTTQQLHKKHDELAVVEDAIDALHGQIDSLHTQLAHL VQQLQW
LQAKHDAIHQAKEVDVETVEVERQRDKHSLAVLEQIQHLLALQQRGNA
FLQVSHKKIEKHLRHHPESNPTQALPQLSTHFDEQRLAEVISKLQTIQAAI
QASYIEDANGEVADKQRYDALIQEIATIRAQTQQQLADAQQALSDA
EASLAQFVQEQGNLQQQIAVNEGILADAQAALAHTIATYEARIQEG
QEALAAINLALDVLQQNQSDLQGVEDFSNAVNAVQAGNSTDAGDD
AGDDSGVEGEAFDNTEKVISSNEGRSAMVHVRVLKYPHNILFTNLTND
LFTYLPKTYNESNFVSNVLEVELNDGELFVLACELINKKCFQEGKEKAL
VKSNKIIVHKNLTIFKAPFYVTSKDVNTECICKFKNNNVKIVLKPKYEK
KVINGCNFSSNVSSKHTFTDSLDISLVDDSANISCNVHLSEPKYNHLVGL
NCPGDIIPDCFFQVYQPESEELEPSNIVYLDSQINIGDIEYYEDAEGDDKI
KLFGIVGSIPKTTSFTCICKKDKNSAVNTVTIDHHHHHH *CPAGTVVDDGT*
STNFVALASECTRCQANFVASKTSGFAAGTIDTCTECSKRLTSGATARVVAEAT
QRAQCASYEYDVEPDVA

Bold Underlined: Grl4 Signal (pre) peptide

Bold Italics Text: Grl4 Pro-domain

Bold Text: Mature Grl4

Plain Text:pfs48/45

Underlined Text: 6 x His tag

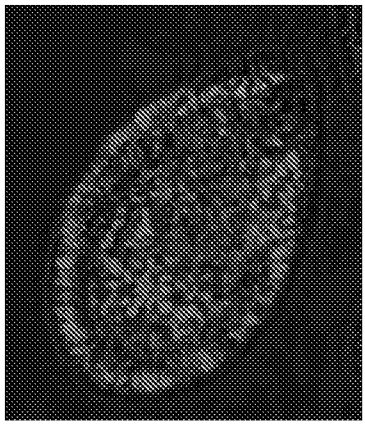
***Italicized Text:* I-Antigen C-terminal domain**

***Bold, Italicized underlined Text*: HA-tag**

Figure 13A

B

Immunofluorescence showing mucocyst localization



C

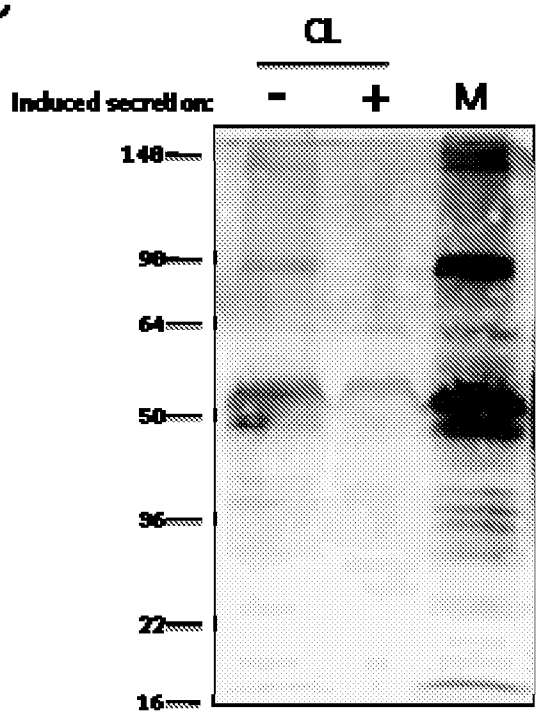
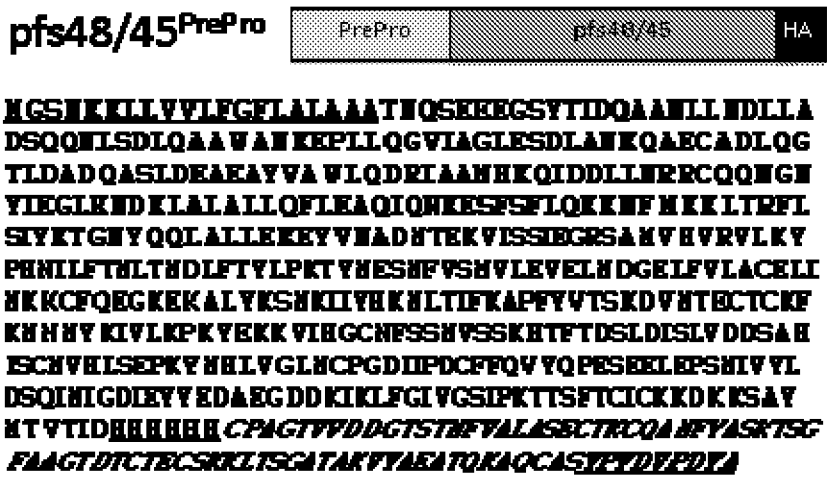


Figure 13B-13C

A



Bold Underlined: Gri1 Signal (ore) peptide

Bold Text: Gri4 Pro-domain

Plain Textpfs48/45

Underlined Text: 6 x His tag

***Italicized Text:* I-Antigen C-terminal domain**

***Bold, Italicized underlined Text*: HA-tag**

Figure 14A

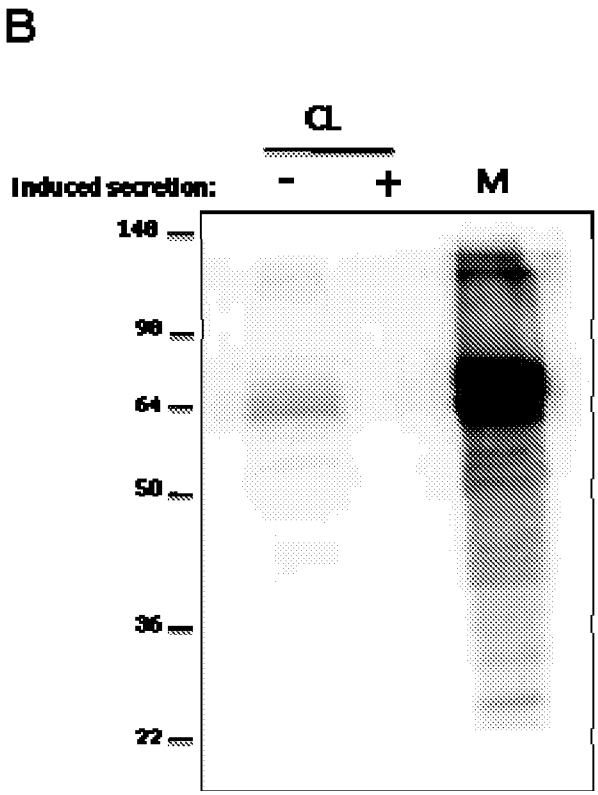


Figure 14B

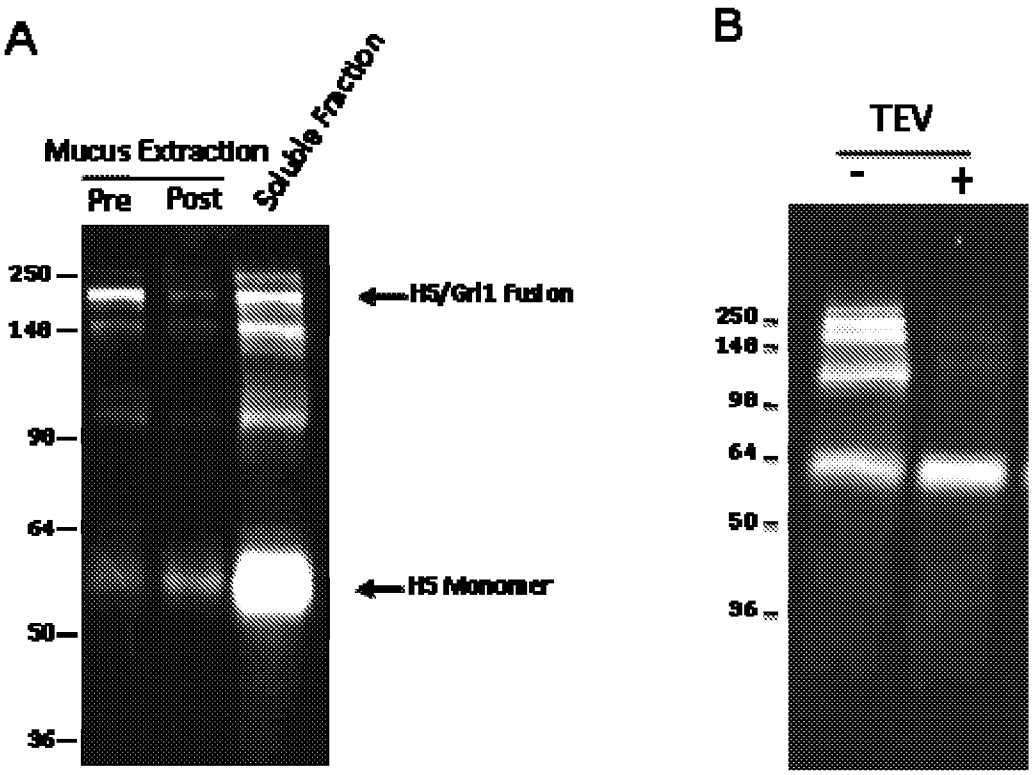


Figure 15A-15B

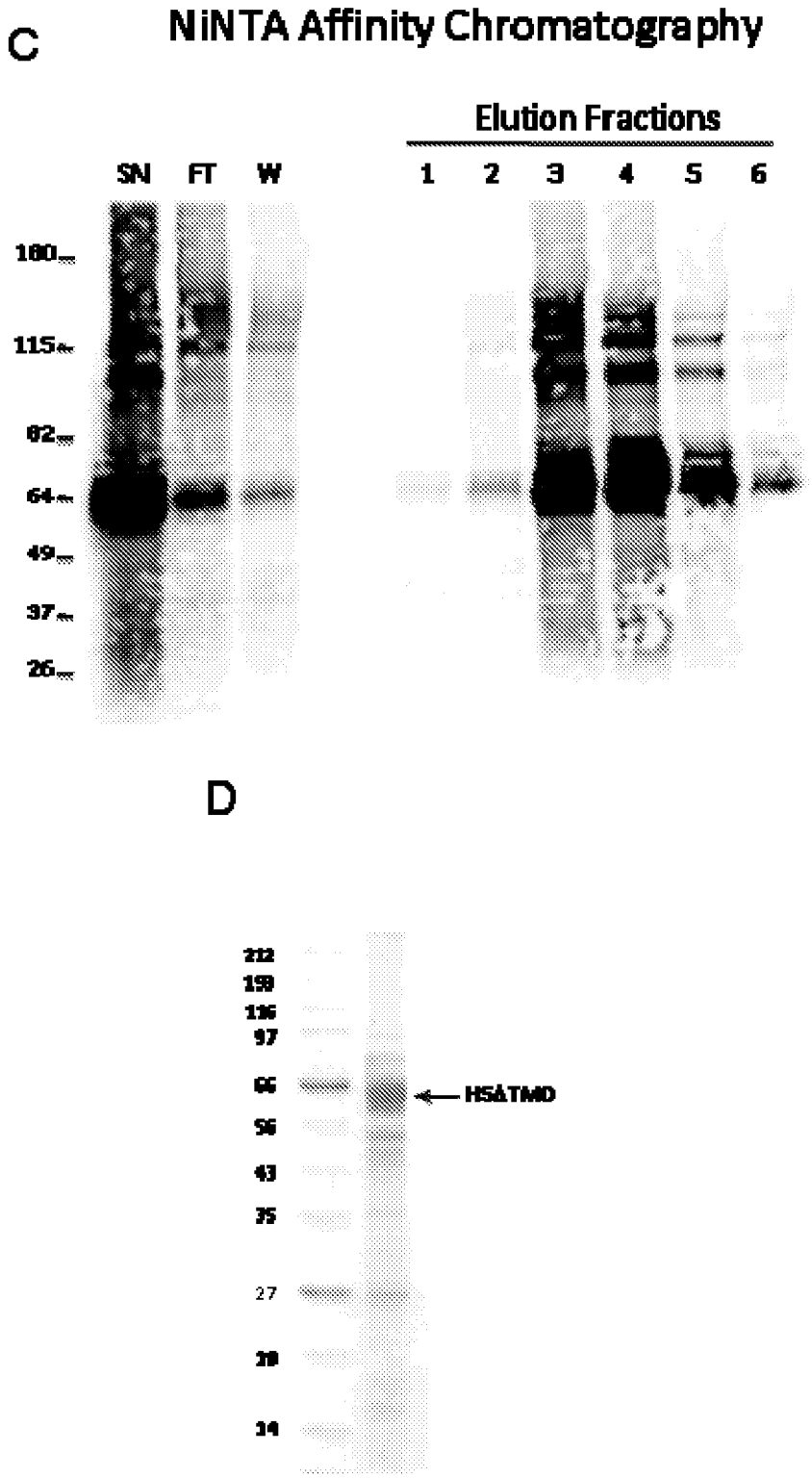


Figure 15C-15D

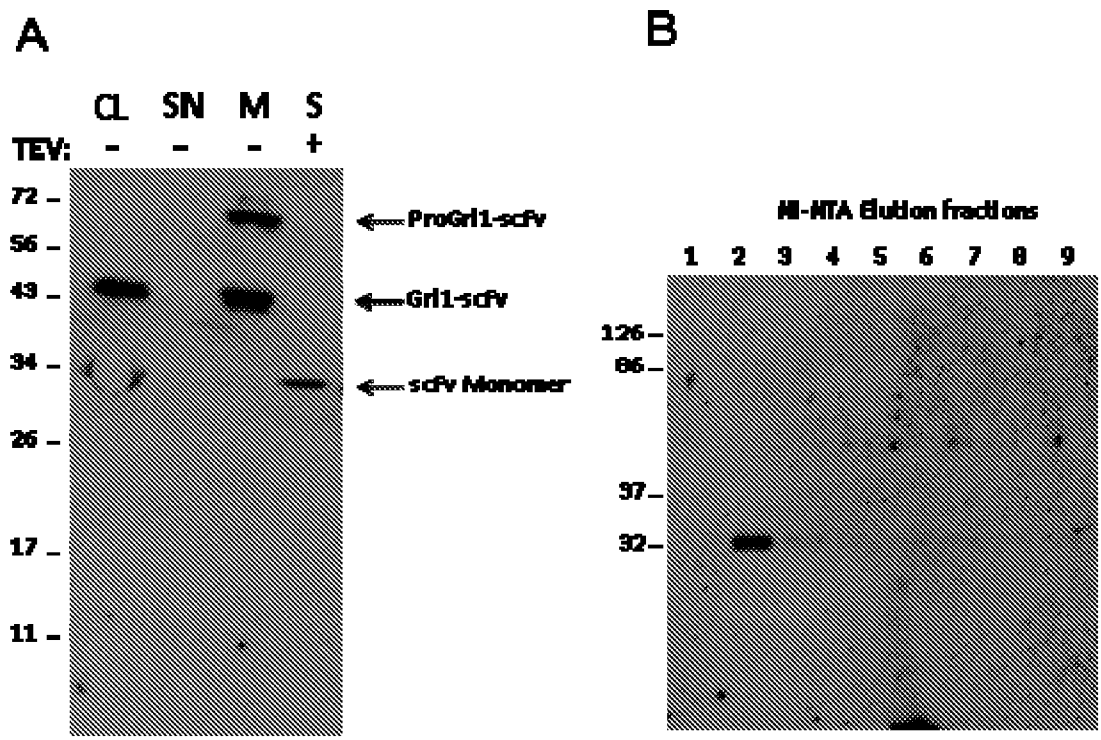


Figure 16A-16B

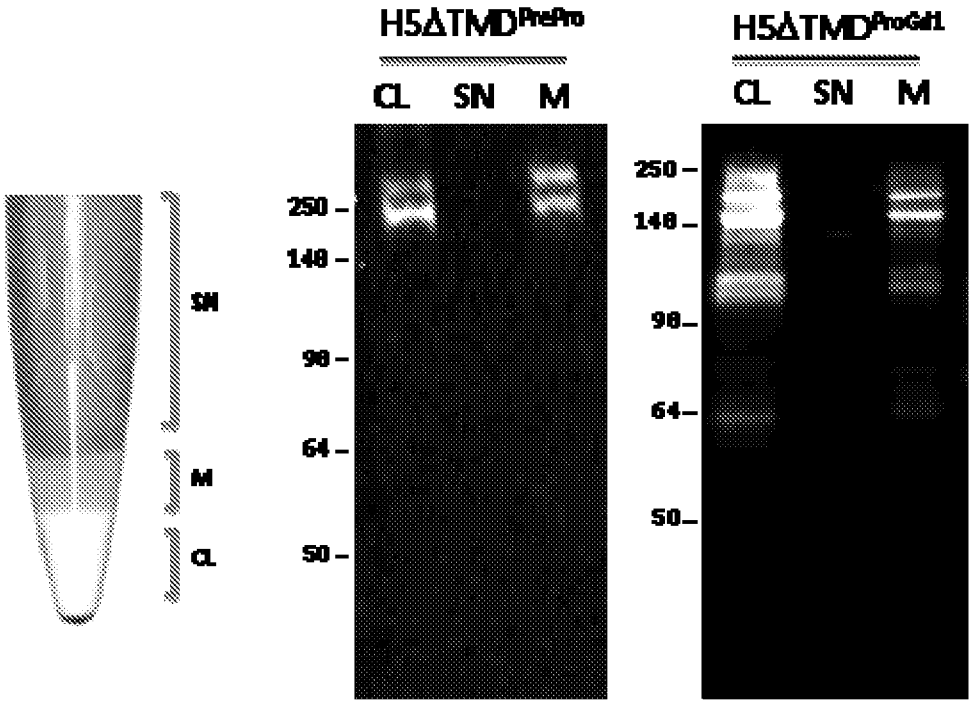

H5ΔTMD^PrePro
CL SN M
250
140
98
64
50
SN
M
CL
H5ΔTMD^ProGal1
CL SN M
250
140
98
64
50

Figure 17

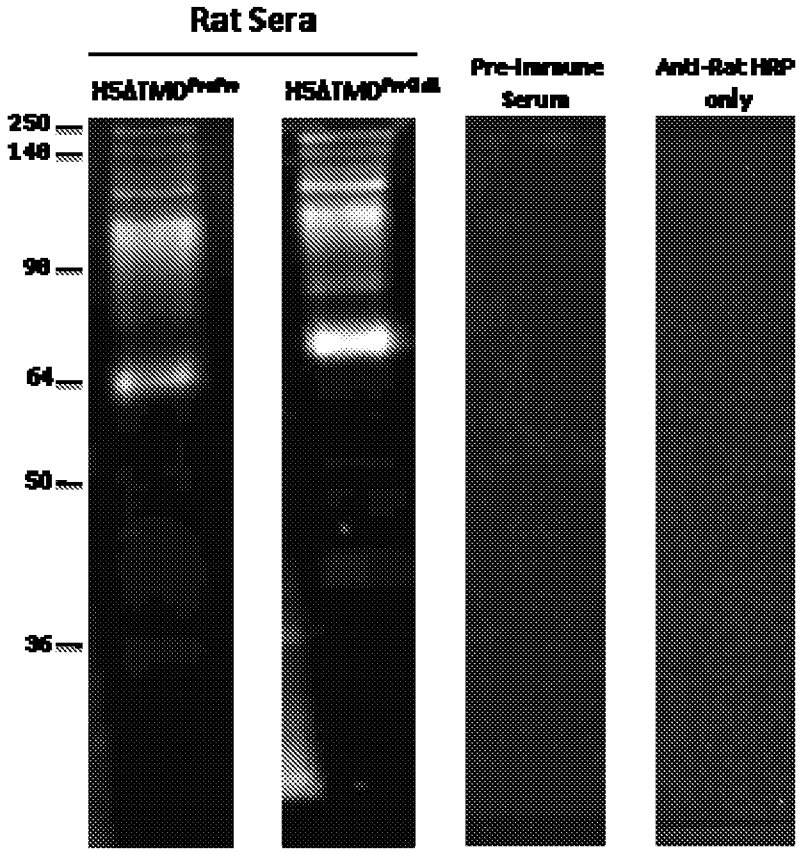


Figure 18