# The α and β Subunits of the Na,K-ATPase Can Assemble at the Plasma Membrane into Functional Enzyme

**Anthony W. DeTomaso, Gustavo Blanco, and Robert W. Mercer**

Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110

*Abstract.* Synthesis and assembly of most oligomeric plasma membrane proteins occurs in the ER. However, the role the ER plays in oligomerization is unknown. We have previously demonstrated that unassociated α and β subunits of the Na,K-ATPase are targeted to the plasma membrane when individually expressed in baculovirus-infected *Sf*-9 cells. This unique property allows us to determine if assembly of these two polypeptides is restricted to the ER, or if it can also occur at the plasma membrane. To investigate the assembly of the Na,K-ATPase we have taken advantage of the ability of baculovirus-infected cells to fuse. Lowering the extracellular pH of the infected cells triggers an endogenously expressed viral protein to initiate plasma membrane fusion. When individual *Sf*-9 cells expressing either the Na,K-ATPase α or β subunits are plated together and subjected to a mild acidic shock, they form large syncytia. In the newly continuous plasma membrane the separate α and β polypeptides associate and assemble into functional Na,K-ATPase molecules. However, a hybrid ATPase molecule consisting of a Na,K-ATPase α subunit and a H,K-ATPase β subunit, which efficiently assembles in the ER of coinfected cells, does not assemble at the plasma membrane of fused cells. When cells expressing the Na,K-ATPase α subunit are fused to cells coexpressing the Na,K-ATPase β subunit and the H,K-ATPase β subunit, the Na,K-ATPase α subunit selectively assembles with the Na,K-ATPase β subunit. However, when cells are coinfected and expressing all three polypeptides, the Na,K-ATPase α subunit assembles with both β subunits in the ER, in what appears to be a random fashion. These experiments demonstrate that assembly between some polypeptides is restricted to the ER, and suggests that the ability of the Na,K-ATPase α and β subunits to leave the ER and assemble at the plasma membrane may represent a novel mechanism of regulation of activity.

ASSEMBLY between individual subunits of oligomeric proteins synthesized in the secretory pathway usually occurs in the ER. Typically the unassociated subunits of these complexes are retained in the ER until properly assembled, after which they traverse the secretory pathway to their final cellular destination (Hurtley and Helenius, 1989; Rose and Doms, 1988). It has been well established that ER resident proteins (e.g., Bip, GRP 94) assist in the folding of polypeptides from a nascent to mature form (Gething and Sambrook, 1992), and that the ER physical environment is conducive to steps in proper folding (Hwang et al., 1992). Moreover, the speed and specificity of assembly suggests that individual subunits must present unique domains that recognize complementary subunits, and therefore must be in at least a partially folded, stable conformation prior to oligomerization. However, once each subunit has properly folded and acquired a stable conformation it is not known if residence in the ER is essential for the assembly of oligomeric complexes.

Address all correspondence to R. W. Mercer, Department of Cell Biology and Physiology, Washington University School of Medicine, 660 South Euclid Street, St. Louis, MO 63110. Ph.: (314) 362-6924. Fax: (314) 362-7463.

Expression studies of the rodent Na,K-ATPase in baculovirus-infected *Sf*-9 cells have provided a unique system to study the assembly of a multimeric protein. The Na,K-ATPase is a heterodimeric protein consisting of a 100-kD, multi-membrane-spanning α subunit, and a smaller, glycosylated type II membrane protein termed the β subunit, both of which are required for normal activity. This enzyme couples the hydrolysis of ATP with the countertransport of Na and K ions, creating the ion gradients typical of most eucaryotic cells. Previous results from baculovirus expression studies have demonstrated that when the α and β subunits are expressed individually in *Sf*-9 cells, both are in stable conformations and unassociated with other proteins. Moreover, the independent α and β subunits are targeted to the plasma membrane (DeTomaso et al., 1993*b*). This finding is in contrast to studies with amphibian or avian Na,K-ATPase molecules, which have suggested that the α and β subunits of the Na,K-ATPase, similar to individual subunits of other oligomeric proteins, are retained in the ER until they are properly assembled (reviewed in Geering, 1991). The targeting of individual Na,K-ATPase subunits to the plasma membrane of insect cells is not a result of the invertebrate ER failing to recognize an unassembled mammalian protein, nor is it an

artifact caused by the baculovirus infection. When *Sf*-9 cells are infected with baculoviruses expressing either murine IgG heavy or light chain polypeptides, the expressed polypeptides are retained intracellularly. Only the fully assembled tetrameric immunoglobulin is secreted in cells expressing both polypeptides. Thus, as has been suggested in several mammalian systems, the individual Na,K-ATPase subunits may exist independently from one another outside the ER (Marxer et al., 1989; Mircheff et al., 1990; Hundal et al., 1992). These observations suggest that there may be unknown physiological or regulatory roles of the independent Na,K-ATPase subunits.

The present study addresses if the two N,K-ATPase subunits retain the ability to assemble at the *Sf*-9 cell plasma membrane, or if assembly can occur only in the ER. Using a baculovirus fusogenic protein, the plasma membranes of cells expressing each subunit can fuse, allowing the two polypeptides to interact. The individual subunits of the Na,K-ATPase can assemble at the plasma membrane of these syncytia. In contrast, a hybrid ATPase consisting of the Na,K-ATPase $\alpha$ subunit and the H,K-ATPase $\beta$ subunit, which efficiently assembles in the ER of the *Sf*-9 cell, cannot assemble at the plasma membrane. Thus, while assembly of the Na,K-ATPase $\alpha$ and $\beta$ subunits can occur at both the ER and the plasma membrane, assembly of the hybrid ATPase is restricted to the ER. This assembly of the Na,K-ATPase at the plasma membrane is specific and may be important in regulating or modifying enzymatic activity.

## Materials and Methods

### Cells and Virus

*Sf*-9 cells were purchased from the American Type Culture Collection (Rockville, MD; CRL 1711). Cells were cultured and infected according to standard procedures (Summers and Smith, 1987). In most cases, cells were infected with each virus at a multiplicity of infection (MOI)[1] of 10. In cells coinfected with all three viruses the Na,K $\alpha$ virus was at a MOI of 5 and the H,K $\beta$ or Na,K $\beta$ viruses were at MOIs of 5 or 10. In coinfections the amount of recombinant protein produced was roughly proportional to the corresponding virus MOI, but this was not a linear relationship (not shown). The baculoviruses corresponding to the rodent Na,K-ATPase $\alpha$1 or $\beta$1 subunits have been previously described (DeTomaso et al., 1993*b*). A recombinant baculovirus corresponding to the rabbit H,K-ATPase $\beta$ subunit was provided by George Sachs (UCLA, Los Angeles, CA). Two recombinant baculoviruses corresponding to the murine IgG heavy and light chains were provided by Dr. Donald J. Capra (University of Texas Southwestern Medical Center, Dallas, TX).

### Antibodies

The $\alpha$-specific monoclonal antibody (C464-6B) was a gift of Dr. Michael Caplan (Yale University School of Medicine, New Haven, CT). A polyclonal antibody against the rodent $\beta$1 subunit was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). A polyclonal antibody against the $\beta$ subunit was provided by Dr. Amir Askari (Medical College of Ohio, Toledo, OH). The mAb to the H,K-ATPase $\beta$ subunit was provided by Dr. J. Forte (University of California, Berkeley, CA). $^{125}$I-labeled secondary antibodies were purchased from ICN Biochemicals (Irvine, CA). Texas red and fluorescein-conjugated secondary antibodies were purchased from Cappel Laboratories (West Chester, PA). IgG light chain was visualized by a rabbit anti-mouse light chain specific primary antibody. IgG heavy chain was visualized with a fluorescein-conjugated Fc-specific goat anti-mouse antibody.

1. *Abbreviations used in this paper*: HBS, Hepes-buffered saline; MOI, multiplicity of infection.

### Cell Fusion

Infected *Sf*-9 cells were fused according to procedures outlined by Leikina et al. (1992). TNM/FH medium (Summers and Smith, 1987) supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, and 0.25 $\mu$g/ml fungizone, pH 6.2 (complete medium) covering confluent monolayers was aspirated and replaced by Grace's medium, pH 5.1 for 5 min at 27°C. After this incubation, the low pH medium was aspirated and replaced by complete medium. To ensure maximal syncytium formation the cells were incubated for 2 h before harvesting.

### Fusion-dependent Assembly Assay

32 h after infection, *Sf*-9 cells individually infected in spinner flasks were gently centrifuged (1,500 *g*) and the media aspirated. The cells were washed by resuspension in 10 ml of complete medium followed by centrifugation. This wash was repeated four times. Cells were then seeded according to the figure legends and allowed to settle for 1 h in six-well tissue culture plates. In all experiments involving ATPase polypeptides, cells were plated at a ratio of one Na,K $\alpha$-infected cell to three Na,K $\beta$, H,K $\beta$ or coinfected cells. This was done to raise the probability that both subunits would be in each syncytium, since the average syncytia was small (four to six cells), and immunoprecipitations were with an anti-Na,K-ATPase $\alpha$ antibody. In experiments involving IgG polypeptides, cells were plated at a 1:1 ratio of heavy chain infected or light chain–infected cells. After cells had settled into a confluent monolayer, complete medium was removed and replaced by methionine-free Grace's medium. After 1 h, 50 $\mu$Ci of [$^{35}$S]methionine were added and cells incubated for 20 min. The radioactive medium was removed, followed by a 2-h incubation in complete medium. This allowed the labeled peptide to transit the biosynthetic pathway to the plasma membrane. The incubation time after labeling was determined from results of cell surface biotinylation experiments (not shown) and from the time required to target all the $\alpha$ and $\beta$ polypeptides from intracellular organelles to the plasma membrane during cycloheximide treatment as assayed by immunofluorescent microscopy (DeTomaso et al., 1993*b*). Unless otherwise stated, cycloheximide (100 $\mu$g/ml; Calbiochem-Behring Corp., San Diego, CA) was added to all media after the labeling period. Baculovirus-directed recombinant protein synthesis is controlled by the polyhedrin promoter which is not maximal until late in the infectious cycle (>40-h after infection). However, these experiments were performed when cell–cell fusion is maximal (36-h after infection), and recombinant protein synthesis is relatively low. Cycloheximide treatment allowed the majority of the expressed ATPase polypeptides at the plasma membrane to be labeled, and ensured that interactions within the syncytia occurred between presynthesized proteins only. This cycloheximide treatment was reversible after a 6-h incubation with little cell death (not shown). Cells were then fused as described and incubated for another 2 h.

### Immunoprecipitations

Metabolically labeled cells were solubilized for 10 min on ice with 1 ml of Hepes-buffered saline (HBS; 150 mM NaCl, 25 mM Hepes, pH 7.4) containing 1% CHAPS. 1 mM *N*-ethylmaleimide (Pierce Corp., Rockford, IL) was included when solubilizing IgG syncytia to block free cysteines and prevent spurious disulfide bond formation. Insoluble material was pelleted for 15 min at 100,000 *g* in a table top ultracentrifuge (TL-100, Beckman Instruments). The supernatant was immunoprecipitated with 50 $\mu$l of either an anti-Na,K $\alpha$ hybridoma supernatant (C464-6B) or 6 $\mu$l of an anti-H,K-ATPase $\beta$ and 50 $\mu$l of goat anti–mouse coated magnetic Dynabeads (Dynal Corp., Great Neck, NY) at 4°C overnight on a rocking table. Immunoprecipitates were washed 3× in CHAPS buffer by isolating the Dynabeads on the side of the microfuge tube with a magnet and aspirating the buffer. After the last wash all the buffer was aspirated. In experiments with IgG, protein was eluted by incubation in 1× Laemmli sample buffer (2% SDS, 100 mM Tris-HCl, pH 6.8, 33% glycerol) ± 100 mM DTT. After 10 min at 65°C the samples were resolved by SDS-PAGE; gels were incubated in 1 M Na salicylate for 1 h, dried, and subjected to fluorography. When digestion of oligosaccharides was necessary, Dynabeads were resuspended in 50 $\mu$l of a solution containing 1% $\beta$-mercaptoethanol, 0.5% SDS and boiled for 10 min to elute the bound protein. After cooling, 5.5 $\mu$l of both 10% NP-40 and 0.5 M sodium phosphate (pH 7.5) were added and the supernatant divided into two tubes. Into one tube 20 U of peptide N-glycanase F (New England Biolabs, Beverley, MA) were added to digest the oligosaccharides. Digestion was for 90 min at 37°C. Undigested samples were also incubated in this manner without added enzyme. After this incubation, 25

μl of 2× Laemmli sample buffer were added to each tube and samples processed for SDS-PAGE as described.

### Immunofluorescence Microscopy

Confocal microscopy and immunofluorescence labeling of Na,K-ATPase α and β subunits, the H,K β subunit, and the IgG heavy and light chain polypeptides were performed as previously described (DeTomaso et al., 1993*b*). Cells seeded on 10-mm round coverslips in 24-well tissue culture plates were incubated as described, then fixed in 2% paraformaldehyde in HBS and permeabilized with 0.5% Triton X-100. Cells were incubated with the corresponding antibodies (see above) overnight at 4°C, washed, and incubated with goat anti-mouse Texas red or goat anti-rabbit fluorescein-conjugated secondary antibodies (Cappel Laboratories, West Chester, PA). Images were analyzed on a Zeiss Axioplan microscope fitted with a Bio-Rad MRC confocal imaging system under a 63× objective.

### Potassium Uptakes

Infected cells were plated in 6-well tissue culture plates as described in the fusion-dependent assembly assays. After a 2-h incubation in cycloheximide, the cells were fused as described above. Fusion and the after fusion incubation also occurred in the presence of cycloheximide. After 2 h, the tissue culture plates were placed in shallow water baths at 37°C. The medium was removed and replaced with a prewarmed (37°C) solution containing 150 mM NaCl, 10 mM KCl, 25 mM Hepes, pH 7.4, 5 mM $MgCl_2$, 1% bovine serum albumin, and 100 μM bumetanide, with or without 1 mM ouabain. The cells were incubated for 5 min, then $^{86}Rb$ was added at 400 cpm/μl. In previous experiments, $^{86}Rb$ uptake was identical to $^{42}K$ uptake (not shown). The cells were then incubated for 3 or 4 min in the presence of the tracer. Uptake was linear for at least 5 min (not shown). The radioactive medium was removed by aspiration and the cells quickly washed three times with 4 ml of ice cold HBS. After the last wash all the medium was aspirated and the cells were allowed to dry for 1 h at room temperature. Cells in each well were solubilized in 0.5 ml of 1% Triton X-100 in HBS. The radioactivity of a 300-μl sample was determined by liquid scintillation. The protein concentration of each well was determined in triplicate using bovine serum albumin as standard and bicinchoninic acid/copper sulfate as described by the supplier (Pierce Chemical Co., Rockford, IL). Uptake was normalized to protein concentration. Each experimental point was the average of six wells, and the data shown in the histogram is the average of three separate experiments ±SEM.

### Sucrose Gradient Velocity Sedimentation

Crude membranes (~100 μg protein) were solubilized in 1% CHAPS in HBS for 1 h at 4°C. Insoluble material was pelleted in a microfuge for 15 min. The supernatant (1 ml) was layered on top of a 11-ml linear sucrose gradient (5–20%) made in HBS and 1% CHAPS and centrifuged for 18 h at 4°C in a SW-41 rotor (Beckman Instruments, Palo Alto, CA) at 40,000 rpm. Sixteen 0.75-ml fractions were collected from the top of the gradient using a collector (Buchler Instruments, Lenexa, KS). The fractions were concentrated following the procedure of Suzuki et al. (1991), which allowed for quantitative recovery of 5 μg protein from 1 ml of 20% sucrose (data not shown). Each fraction was brought to 1 ml by the addition of $dH_2O$, followed by the addition of 100 μl of 0.15% deoxycholate. After 10 min at room temperature 100 μl of 72% TCA was added and the fractions were incubated on ice for 30 min. The fractions were centrifuged in a microfuge for 15 min and the supernatant removed by aspiration. The pellet was neutralized with $NH_4OH$ gas and resuspended in SDS-PAGE sample buffer. After a 15-min incubation at 65°C the samples were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the indicated antibodies. Apparent sedimentation coefficients of the α and β polypeptides were obtained by interpolation of a standard curve (*S* determined at 20°C in water of 4.3, bovine serum albumin; 7.6, yeast alcohol dehydrogenase and 11.3, bovine liver catalase; $r = 0.99$) analyzed on a separate gradient.

### Quantification of Polypeptides Using Densitometry

Autoradiographs were digitized using an Epson ES-300C scanner interfaced to a Macintosh IIci computer. Intensity of the bands was determined using the NIH Image 1.52 software. Multiple exposures were analyzed to ensure that the signals were within the linear range of the film.

## Results

### The Unassociated Na,K-ATPase α and β Subunits Exist in Stable Conformations at the Plasma Membrane of the Infected *Sf*-9 Cell

Several lines of evidence suggest that the unassociated α and β subunits of the heterodimeric Na,K-ATPase exist in stable conformations when expressed individually in baculovirus-infected *Sf*-9 cells. Fig. 1 shows representative fields of α- and β-infected *Sf*-9 cells analyzed by immunofluorescent confocal microscopy 36 h after infection. At this time in the baculovirus infectious cycle, ~30% of the cells exhibit high levels of expression, while the majority of the remaining cells show slight immunoreactivity. As we have previously shown, when each subunit is expressed alone, the majority of the protein is located at the plasma membrane (DeTomaso et al., 1993*b*).

The relevance of targeting unassembled α and β polypeptides to the plasma membrane in infected *Sf*-9 cells is unknown, but does demonstrate that each subunit can be transported out of the endoplasmic reticulum. Thus each unassociated subunit exists in a stable conformation and not as a series of denatured, insoluble aggregates inaccessible to the sorting machinery of the secretory pathway. This is demonstrated in Fig. 1. Detergent-solubilized membranes from cells infected with either the α or β baculovirus were resolved by velocity sedimentation on 5–20% sucrose gradients. Each subunit resolved into a distinctive peak, the α subunit at 5 *S* (Fig. 1 *C*), and the β subunit at ~4 *S* (Fig. 1 *D*). The free α and β subunits are not associated with other proteins and can be separated from the αβ heterodimer (9.5 *S*) in coinfected cells (DeTomaso et al., 1993*b*). Furthermore, we have recently shown that the unassociated α subunit mediates a ouabain-insensitive ATPase activity not present in the αβ heterodimer, providing further evidence that the α subunit exists as a stable entity when unassociated with the β subunit (DeTomaso et al., 1993*a*). Consequently, in infected *Sf*-9 cells it appears that the unassembled α and β polypeptides can exist in stable conformations at the plasma membrane.

### Infected *Sf*-9 Cells Can Form Syncytia

To explore the possibility that unassociated Na,K-ATPase α and β subunits can assemble at the plasma membrane, we have taken advantage of a property of the baculovirus expression system: acidic pH triggers syncytia formation between infected *Sf*-9 cells. An endogenously expressed viral protein, gp64, is an envelope glycoprotein that is sufficient to mediate pH-dependent membrane fusion (Blissard et al., 1992). The likely function of gp64 is to promote viral entry from the host endosome into the cytoplasm. During the baculovirus infectious cycle, nascent viruses bud from the cells 12 to 48 h after infection. The gp64 protein is at the cell surface during this period of viral budding, and if infected *Sf*-9 cells are exposed to a pH of 5.5 or lower, latent gp64-dependent membrane fusion activity is initiated and syncytia formation ensues (Leikina et al., 1992; Blissard et al., 1992). Maximum fusion activity and recombinant protein synthesis are not concurrent; maximum fusion occurs 36–48 h after infection and declines after this time, while recombinant proteins are not maximally expressed until at least 48 h after infec-

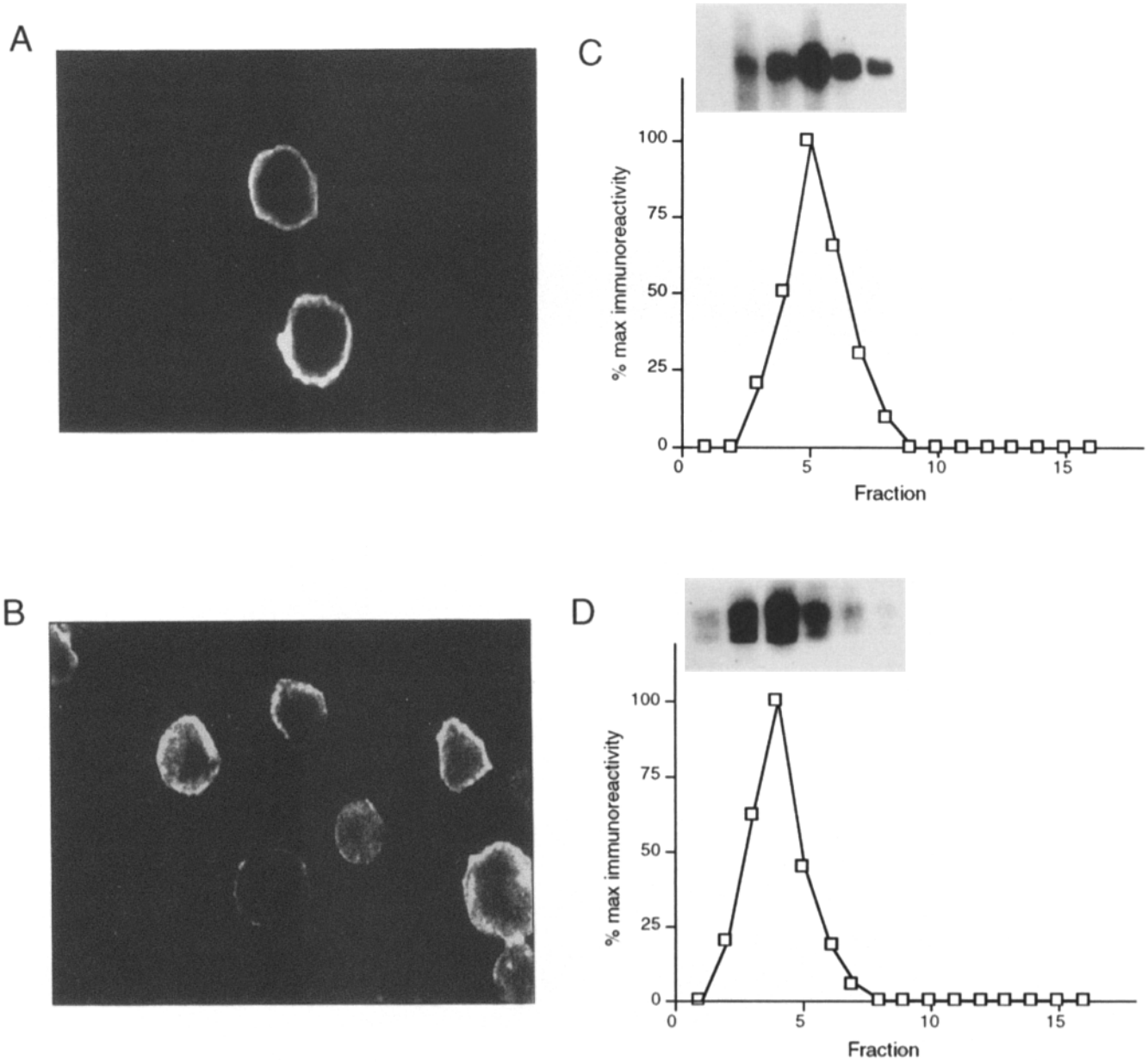
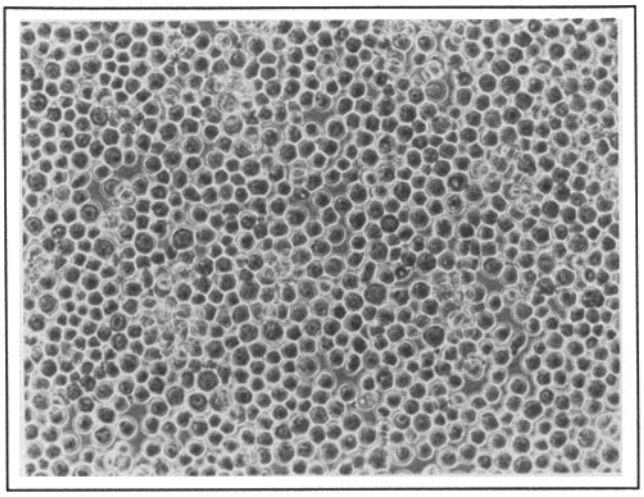



*Figure 1.* Unassociated Na,K-ATPase $\alpha$ and $\beta$ subunits are in stable conformations at the plasma membrane of baculovirus-infected *Sf*-9 cells. *Sf*-9 cells in spinner flasks were individually infected with recombinant baculoviruses expressing either the Na,K-ATPase $\alpha$ or $\beta$ subunits. 35 h after infection a portion of the cells were plated on cover slides and allowed to settle for 1 h. The cells were fixed and the distribution of the expressed polypeptides analyzed by immunofluorescent confocal microscopy. (*A*) The $\alpha$ subunit was identified with an $\alpha$-specific monoclonal antibody followed by Texas red–conjugated goat anti–mouse secondary antibody. (*B*) The $\beta$ subunit was identified with a $\beta$-specific polyclonal antiserum followed by a FITC-conjugated anti-rabbit secondary antibody. Crude membranes from the remaining cells were solubilized in 1% CHAPS and resolved by velocity sedimentation on 5–20% sucrose gradients. Fractions were concentrated by TCA precipitation, resolved by SDS-PAGE, transferred to nitrocellulose and identified with the same primary antisera as in *A* and *B* above. (*C*) Membranes resolved from the $\alpha$ infected cells; and (*D*) from the $\beta$-infected cells. Both polypeptides sediment to characteristic peaks and can be resolved from the $\alpha\beta$ heterodimer (not shown).

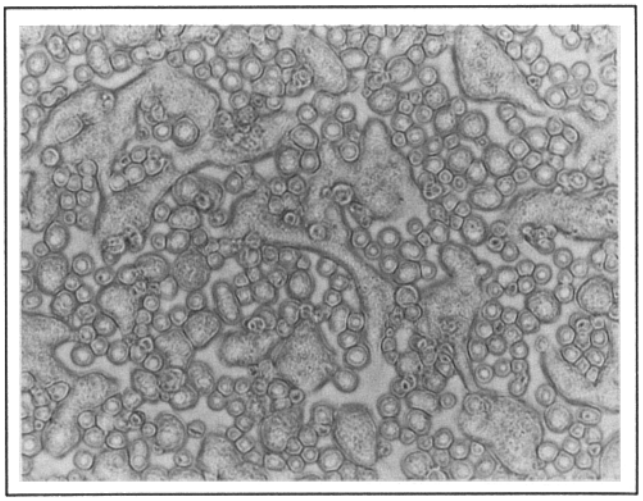
tion. However, as shown in Fig. 1, recombinant protein expression 36–38 h after infection are quite substantial. Typical syncytium formation 36 h after infection under our experimental conditions is shown in Fig. 2. 50 to 70% of the cells are involved in syncytia consisting of 2 to >200 cells, with an average syncytium consisting of four to six cells.

### *Fusion Does Not Affect the Intracellular Compartment*

Initially, we wanted to characterize the effects of fusion on individual cells. Specifically, is syncytium formation limited to fusion of the plasma membranes, or does cell fusion in-



Non Fused Sf-9 Cells



Fused Sf-9 Cells

*Figure 2.* Baculovirus-infected *Sf*-9 cells can form syncytia. *Sf*-9 cells infected in spinner flasks were plated 32 h after infection and allowed to settle for 4 h. Syncytia formation was initiated as described in Materials and Methods. Under these experimental conditions, between 60–70% of the cells are involved in syncytia consisting of 2 to >200 cells, with the average syncytium of four to six cells.

clude integration of the secretory pathways of the individual cells within the syncytium? Because the majority of the Na,K-ATPase $\alpha$ and $\beta$ subunits are at the plasma membrane when fusion occurs (Fig. 1, *A* and *B*), characterization of the redistribution of these subunits upon syncytium formation will not elucidate intracellular processes. Therefore we examined syncytia formed between cells expressing either the IgG heavy or light chain polypeptides. These proteins are ideal for characterizing the intracellular effects of syncytium formation because they are mainly retained in the ER when independently expressed. The heavy chain distribution remains almost completely intracellular (>97%), and the light chain polypeptides, though mostly in the ER, are slowly secreted with a $t_{1/2}$ of 12–16 h. The heavy chain is only secreted when part of a properly assembled $H_2L_2$ tetramer

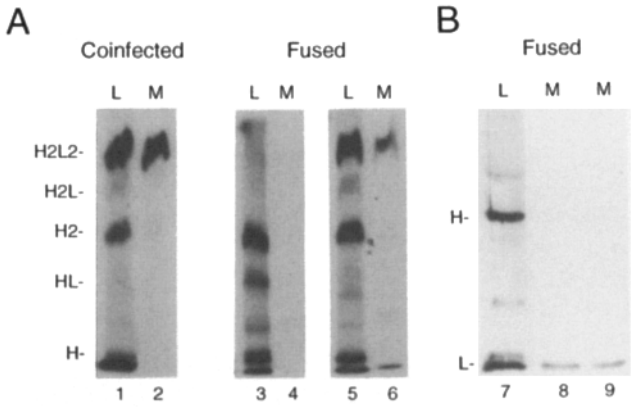



*Figure 3.* Presynthesized murine IgG heavy and light chains do not assemble in fused cells. *Sf*-9 cells were coinfected with baculoviruses expressing the murine IgG heavy or light chain polypeptides, or individually infected with each virus, and metabolically labeled. Cells individually infected with each virus were then fused in the presence or absence of cycloheximide. Both the cell lysate (*L*), and overlaying medium (*M*) were immunoprecipitated. (*A*) Immunoprecipitates from coinfected (lanes *1* and *2*) and individually infected fused (lanes *3–6*) cells were resolved on unreduced 5% gels to distinguish partially and fully assembled IgG oligomers. Individually infected cells were fused in the presence (lanes *3* and *4*) or absence (lanes *5* and *6*) of cycloheximide. (*B*) Immunoprecipitates from fused cell lysates, the before, and the after fusion medium (lanes *7–9*) were reduced and resolved on a 10% gel. Cycloheximide was present during fusion.

with a $t_{1/2}$ of about 4 h (DeTomaso et al., 1993*b*). Additionally, it has been demonstrated in *Xenopus* oocytes, cultured at 18°C, that assembly of murine IgG heavy and light chains can occur posttranslationally, even when the two polypeptides are synthesized over 24-h apart (Colman et al., 1982). Thus, by fusing cells individually infected with either the IgG heavy or light chains it should be possible to determine if there is overlap in the secretory pathways of cells within the syncytium. The distribution of the polypeptides inside a syncytium can be easily monitored by immunofluorescence. Furthermore, by assaying for assembly of the presynthesized heavy and light chains into $H_2L_2$ tetramers we can determine if the unassembled immunoglobulins meet in an ER within the syncytium. For instance, if ER retention of polypeptides is a dynamic process in which the unassembled polypeptides are constantly shuttling between the ER and the *cis*-Golgi or intermediate compartment, then the transport vesicles originating from adjacent cells might mix, allowing IgG heavy and light chains to associate and assemble. This mechanism of ER retention has been suggested for MHC class I molecules (Hsu et al., 1991), and intercellular mixing of proteins between individual ER and Golgi has been previously demonstrated within CHO cell syncytia (Rothman et al., 1984). Moreover, it has been suggested that higher order assembly of hepatitis B surface antigen polypeptides occurs in an intermediate compartment between the ER and the Golgi apparatus (Huovila et al., 1992). Therefore, if the heavy and light chain polypeptides meet in this compartment, they may be able to associate and assemble.

To examine these possibilities, *Sf*-9 cells individually infected with baculoviruses corresponding to murine $\mu$ heavy

chain or κ light chain were assayed for fusion-dependent assembly as described. Briefly, the individually infected *Sf*-9 cells were plated together and proteins were metabolically labeled with [$^{35}$S]methionine, followed by a 2-h chase period. Extracellular pH was lowered for 5 min and syncytia allowed to form for 2 h. The chase, acidic pH pulse and syncytia formation all occurred in the presence of cycloheximide. Cycloheximide allows a large percentage of the recombinant protein to be labeled and it prevents the mRNA suddenly made available to all the cells in the newly formed syncytia from being translated, ensuring that observed interactions are only among proteins synthesized prior to fusion. Following the after fusion incubation period, both the media and cell lysates were immunoprecipitated with anti-mouse IgG secondary antibodies. Immunoprecipitates were eluted and resolved by SDS-PAGE. One half of the sample was left unreduced and resolved on a 5% gel to separate the native immunoglobulin forms, the other half was reduced and separated on a 10% gel (Fig. 3).

The first two lanes in Fig. 3 *A* show immunoprecipitates from the medium and cell lysate of cells coinfected with both the heavy and light chain viruses. The intracellular heavy chain is found in four distinct forms: a fully assembled $H_2L_2$ tetramer yet to be secreted, a partially assembled $H_2L$ multimer and $H_2$ dimer, and as a free monomer (lane *1*). Only the fully assembled tetramer is secreted into the medium (lane 2). In contrast, in fused cells (lanes *3* and *4*) there are no fully assembled tetramers nor heavy and light assembly intermediates (HL, $H_2L$) in the cell lysate or medium. Lanes *7–9* show the reduced samples from the fused cell lysate, the before and the after-fusion medium, respectively. High expression of both the heavy and light chain polypeptides are detected in the fused cell lysate (lane *7*), but only the light chain is immunoprecipitated from the medium both before and after fusion (lanes *8* and *9*). As shown, the fused cells of the syncytium continue to retain the heavy chain intracellularly and slowly secrete the light chain. Thus it appears that the secretory pathways of individual cells within the syncytium are unperturbed by fusion.

A different pattern is seen if cycloheximide is omitted. If protein synthesis is allowed to continue during and after fusion, the heavy and light chain mRNAs redistribute and are translated in other ERs of the syncytium. This allows the formation of fully and partially assembled HL oligomers (Fig. 3 *A*, lanes *5* and *6*). Thus the presynthesized proteins retain the ability to assemble when presented with their complementary subunits, and can then be secreted. Consequently, the absence of HL oligomers in translation-inhibited syncytia demonstrates that the two IgG subunits are not coming in contact with one another.

Two heavy and light chain syncytia analogous to those used in the metabolic labeling experiment are shown in Fig. 4. Immunofluorescent staining confirms the conclusion from

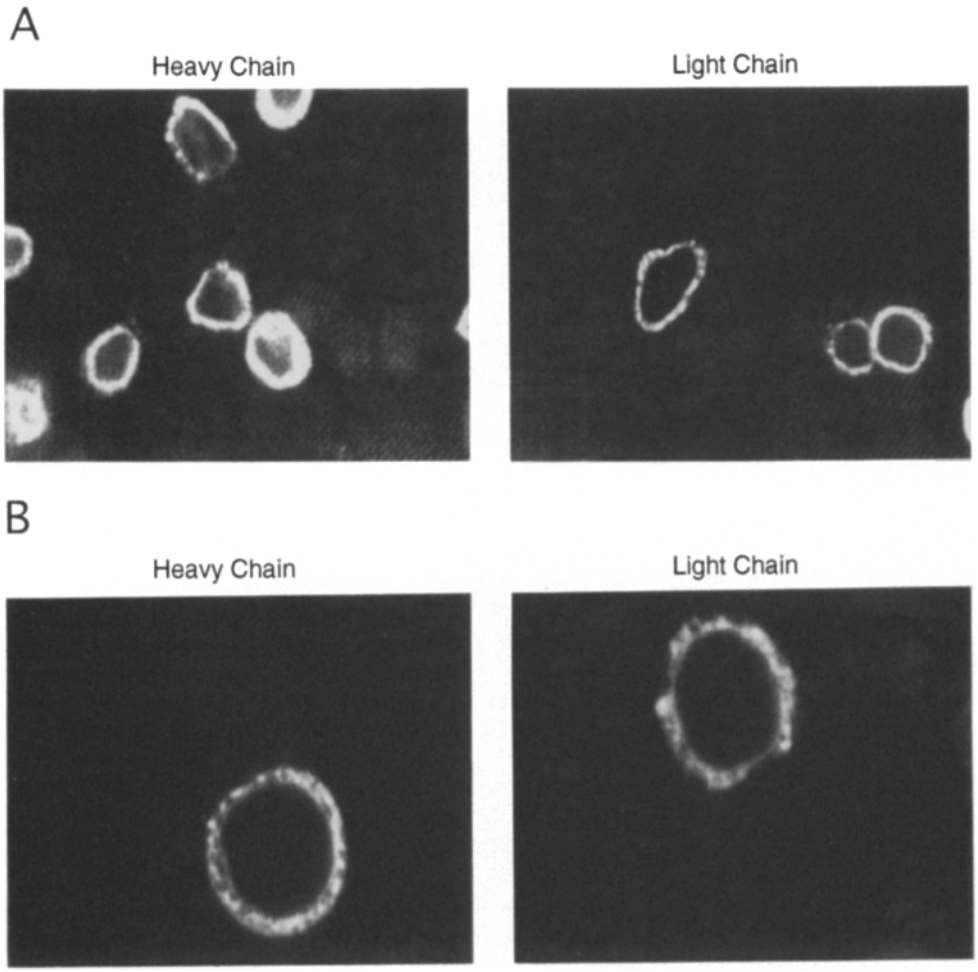



*Figure 4.* Retained IgG heavy and light chains do not redistribute to other ERs within syncytia. The distribution of heavy and light chain polypeptides in a syncytia of over 10 cells (*A*) and of only two cells (*B*) was analyzed by double-labeled confocal microscopy. In both syncytia translation was inhibited by the addition of cycloheximide after fusion. The heavy chain was identified using a goat anti-mouse Fc-specific fluorescein-conjugated antibody (*left*). The light chain was identified with a rabbit anti-mouse light chain specific primary antibody followed by a Texas red–conjugated anti-rabbit secondary antibody (*right*).

the pulse–chase experiments that the IgG polypeptides in individually infected cells within a syncytium remain completely autonomous. Although the syncytium in the bottom panel was chased an additional 4 h there is no cellular overlap in the intracellular labeling. This demonstrates that the cytoplasmic constituents do not freely mix within the syncytia; even the light chain in the process of being secreted is not seen in the heavy chain–infected cell during this long incubation period (Fig. 4, *bottom*). These experiments demonstrate that fusion occurs exclusively at the plasma membrane and that within syncytia individual secretory pathways are not perturbed and do not overlap during the time course of these experiments.

### *Individual Na,K-ATPase α and β Subunits Assemble into Functional Heterodimers at the Plasma Membrane*

To determine whether unassociated α and β subunits of the Na,K-ATPase can assemble at the plasma membrane of the α+β syncytia, cells individually infected with each virus were plated together, metabolically labeled and fused exactly as in the previous experiments. Following the after fusion incubation period, syncytia were detergent solubilized and immunoprecipitated with an anti-α–specific monoclonal antibody. Fig. 5 *A* shows immunoprecipitations from αβ coinfected cells, and fused and nonfused adjacently seeded α- and β-infected cells, respectively. As shown, in the syncytia from fused cells the previously synthesized α and β subunits assemble into detergent resistant complexes that are coimmunoprecipitated (Fig. 5 *A*, lanes *3*, and *4*) Half of each immunoprecipitate was digested with glycan F to condense the glycosylated β subunit into a single polypeptide. Incomplete and inefficient glycosylation of proteins is characteristic of insect cells. The expressed β subunit is spread among four molecular weight species corresponding to the core peptide and differential high-mannose type glycosylation of its three *N*-linked glycosylation sites (DeTomaso et al., 1993*b*).

Assembly is a fusion-dependent process. Immunoprecipitates from nonfused, adjacent α- and β-infected cells, do not contain the β subunit, demonstrating that adjacent cells are not detectably coinfecting each other during the time course of this experiment (lanes *5* and *6*). Because protein synthesis is inhibited during and after fusion with cycloheximide, assembly must be occurring between presynthesized subunits. Assembly was maximal in the syncytia two hours after the onset of fusion. To illustrate normal assembly of the subunits in the ER (lanes *1* and *2*), proteins from cells coinfected with both the α and β baculoviruses were labeled for 10 min, chased for 30 min, and then immunoprecipitated. The as-

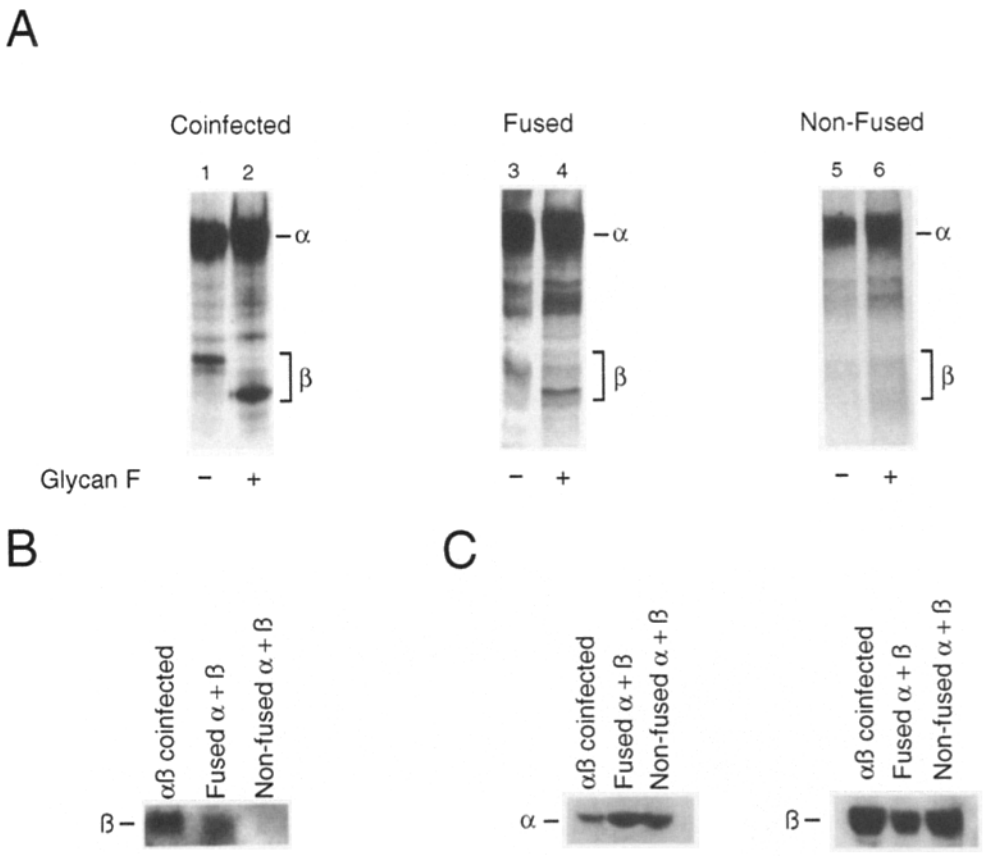



*Figure 5.* Na,K-ATPase α and β subunits assemble in fused *Sf*-9 cells. (*A*) 32 h after infection, *Sf*-9 cells individually infected with the α or β baculoviruses were extensively washed and seeded together in six-well tissue culture plates. Cells were metabolically labeled and fused as described in the Materials and Methods. Following 2 h after fusion incubation period cells were solubilized and immunoprecipitated with an anti-Na,K α mAb. Immunoprecipitates were divided into two, half of each was digested with glycan F to condense the differentially glycosylated β subunit into one band. Immunoprecipitates were then separated by SDS-PAGE and subjected to fluorography. Immunoprecipitates from αβ coinfected cells with a short chase period (20 min) in the absence (lane *1*) and presence (lane *2*) of glycan F. Immunoprecipitates from α + β fused cells in the absence (lane *3*) and presence (lane *4*) of glycan F. Immunoprecipitates from adjacent but unfused α- and β-infected cells in the absence (lane *5*) and presence (lane *6*) of glycan F. (*B*) Cells were infected and fused as in *A* except cells were not metabolically labeled. Proteins from adjacent but unfused α- and β-infected cells (*Nonfused* α + β), α + β fused cells (*Fused* α + *b*), and αβ coinfected cells (αβ) were immunoprecipitated with an anti-Na,K α mAb. The immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose and probed with an anti-β polyclonal antibody. (*C*) Expression of the α and β subunit in the infected cells. Proteins from cells in *B* were probed with the anti-Na,K α and anti-β antibodies.

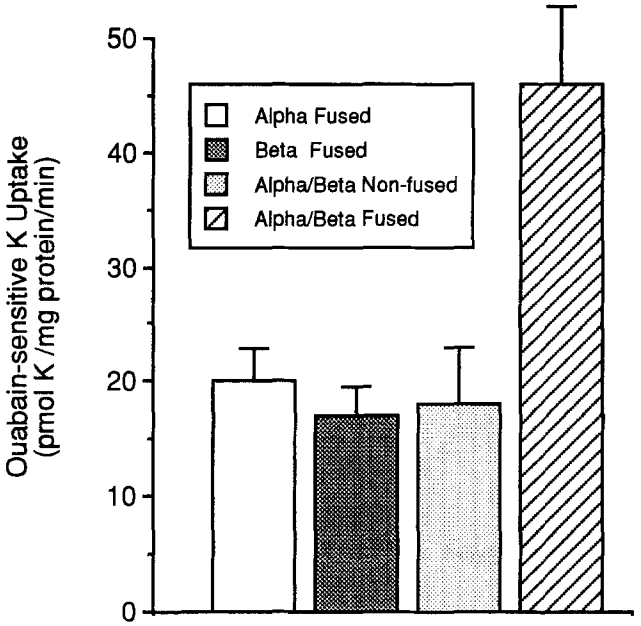



*Figure 6.* Newly formed Na,K-ATPase $\alpha\beta$ heterodimers are functional. Fused and nonfused cells were assayed for ouabain-sensitive potassium uptake as described. Each experimental point is the average of six wells, and the data shown in the histogram is the mean of three separate experiments ±SEM.

sociation of the $\alpha$ and $\beta$ subunits in the ER is a rapid event and is maximal after a 30-min chase. Verification that the coimmunoprecipitated polypeptide is the $\beta$ subunit is provided in Fig. 5 *B*. Cells were infected, fused, and immunoprecipitated with the anti-$\alpha$ mAb as before. However, immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose and probed with an antibody to the $\beta$ subunit. As shown, when the $\alpha$ subunit is immunoprecipitated, the $\beta$ subunit is only identified in the coinfected and individually infected $\alpha$ and $\beta$ cells that have been fused.

If proper assembly of $\alpha\beta$ heterodimers occurs after fusion, then syncytia should show an increase in Na,K-ATPase enzymatic activity. To test for functional activity and to localize the nascent Na pump molecules, both nonfused and fused cells were assayed for ouabain-sensitive potassium uptake. The cardiotonic steroid, ouabain, is a specific inhibitor of the Na,K-ATPase. Cells infected with either the $\alpha$ or $\beta$ baculovirus were plated alone, or together, and incubated for 20 min. Cycloheximide was added, and after 2 h the cells were fused, incubated for another 2 h, and then assayed for ouabain-sensitive potassium uptake (Fig. 6). When $\alpha$- and $\beta$-infected cells are seeded together and not fused, ouabain-sensitive potassium uptake is the same as when these same cells are seeded separately, providing further evidence that cells do not detectably coinfect each other during the incubation periods. There is also no change in ouabain-sensitive potassium uptake when cells infected with either the $\alpha$ or $\beta$ baculovirus alone are fused, demonstrating that cell fusion does not stimulate endogenous *Sf*-9 Na,K-ATPase activity, nor permit aberrant activity of individual $\alpha$ or $\beta$ polypeptides. However, if adjacent $\alpha$- and $\beta$-infected cells are fused, there is a significant increase in ouabain-sensitive potassium uptake compared to individually infected or nonfused cells.

Syncytia formed as above were fixed after the after fusion incubation period and analyzed by confocal microscopy (Fig. 7). The bottom two panels show a four cell syncytium expressing high amounts of both $\alpha$ (*E*) and $\beta$ (*F*) polypeptides. As shown, proteins synthesized before cycloheximide treatment are transported to the plasma membrane, and upon fusion redistribute around the entire syncytium. The only overlap of the two polypeptides is at the plasma membrane, and intracellular colocalization is not observed. The top two panels show syncytia from cells individually infected with the $\alpha$ (*A*) or $\beta$ (*B*) viruses. There are no significant differences in redistribution of polypeptides in syncytia formed with the $\alpha$- or $\beta$-infected cells, nor does the larger $\alpha$ subunit show any less mobility in the membrane.

To further analyze the redistribution of polypeptides after fusion, cells infected with the $\alpha$ virus were fused to cells expressing the murine IgG light chain. The middle two panels (Fig. 7, *C* and *D*) show double label confocal analysis of one of these syncytia. Fig. 7 *C* shows the distribution of the $\alpha$ polypeptides, while *D* shows the light chain polypeptides. In this experiment cycloheximide was not added until after fusion, and cells were also fixed after only a 1.5-h incubation. This permitted protein from the $\alpha$-infected cell to be dispersed throughout the secretory pathway. This distribution is similar to that of the light chain in the light chain infected cell. However, after fusion the intracellular $\alpha$ polypeptides remain in their source cell, as do the light chain polypeptides. Only the $\alpha$ polypeptides at the plasma membrane redistribute to the light chain infected cell. These polypeptides remain at the plasma membrane and are not internalized into the light chain–infected cell. This confirms results from the IgG experiments (Figs. 3 and 4) demonstrating that the intracellular polypeptides remain in their source cell and do not redistribute to other cells within the syncytium. Moreover, it appears that proteins at the plasma membrane are not being endocytosed to a significant degree.

Previous experiments relied on the action of cycloheximide to inhibit translation of proteins after the fusion of adjacent cells, ensuring that interactions are among proteins synthesized before fusion. To illustrate the action of cycloheximide, $\alpha+\beta$ syncytia formed as above but in the absence of the translation inhibitor are show in Fig. 8. As visualized by the heavy ER staining, it appears as if one cell on each side of the syncytium is heavily expressing $\alpha$ or $\beta$ polypeptides. The polypeptides that reach the plasma membrane have been redistributed around the entire syncytium, although in this image the high amount of intracellular staining lowers the contrast available. Quite surprisingly, the distribution of the intracellular $\alpha$ and $\beta$ polypeptides synthesized after fusion and subsequent two hour incubation was not homogeneous, suggesting that mRNA redistribution did not reach equilibrium. It is possible that translation is inhibited after fusion, but it does not seem likely since individual cells are expressing different levels of each protein. Although only indirectly tested by the appearance of the translated polypeptide, it appears that slow redistribution of mRNA occurs within the new intracellular volume of the syncytium. This is a surprising observation considering that the recombinant message inside highly infected cells can account for 30% of the mRNA (Summers and Smith, 1988). More importantly, it is clear from comparing the intracellular staining between syncytia formed in the presence (Fig. 7), or absence (Fig. 8)

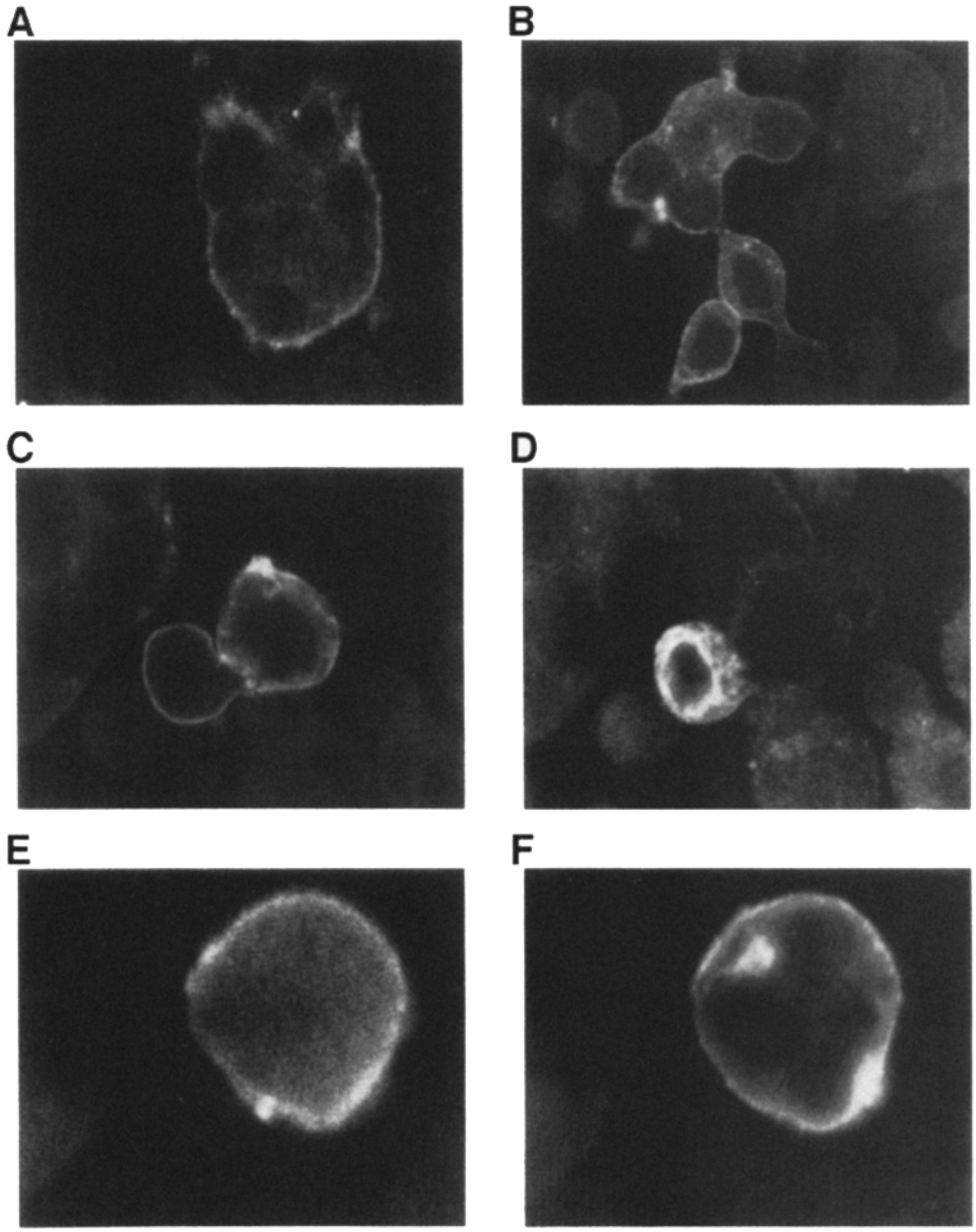



*Figure 7.* Location of polypeptides in syncytia analyzed by immunofluorescence confocal microscopy. Syncytia were fixed, permeabilized, and immunolabeled as described. (*A*) The $\alpha$ subunit in syncytia formed from cells expressing the Na,K-ATPase $\alpha$ subunit. (*B*) The $\beta$ subunit in syncytia formed from cells expressing the Na,K-ATPase $\beta$ subunit. No differences in the redistribution of either polypeptide after fusion were observed. Double-labeled confocal analysis of syncytia formed between cells expressing the Na,K-ATPase $\alpha$ subunit and cells expressing the IgG light chain. (*C*) The distribution of the $\alpha$ polypeptides after fusion. (*D*) The distribution of the IgG light chain polypeptides. Double-labeled confocal analysis of syncytia formed between cells expressing the Na,K-ATPase $\alpha$ or $\beta$ subunits. (*E*) Distribution of the $\alpha$ subunit. (*F*) Distribution of the $\beta$ subunit.

of cycloheximide that treatment is blocking protein synthesis in the syncytia after fusion.

These experiments demonstrate that Na,K-ATPase $\alpha$ and $\beta$ subunits located at the plasma membrane of infected *Sf*-9 cells can assemble into functional heterodimers. This is not a result of cross-infection during incubation periods, nor is it dependent on de novo protein synthesis. In addition, assembly of the subunits is not occurring intracellularly. Results from IgG fusions (Figs. 3 and 4) demonstrate that proteins within the secretory pathways of individual cells do not redistribute within a syncytium, and Fig. 7 demonstrates that there is no intracellular colocalization of polypeptides as a result of endocytosis. Thus the individual subunits of the Na,K-ATPase can assemble outside the endoplasmic reticulum and do so at the plasma membrane.

### *A Hybrid ATPase Can Assemble in the ER But Not at the Plasma Membrane*

Several hypotheses may account for fusion-dependent assembly of Na,K-ATPase $\alpha$ and $\beta$ subunits at the plasma membrane. One is that the assembly of multisubunit proteins is not restricted to the ER. In this case the function of the ER is first to assist in nascent polypeptide folding, then to recognize and retain incompletely folded and assembled polypeptides, preventing their possible deleterious effects elsewhere in the cell. After polypeptides have folded correctly, domains that can interact with complementary subunits are properly presented. At this point assembly may resemble any number of other specific protein interactions that occur outside the ER.

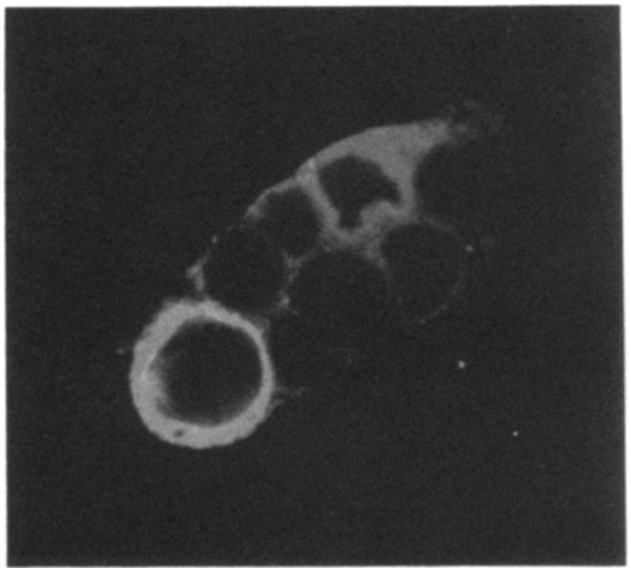


*Figure 8.* Distribution of polypeptides in syncytia not treated with cycloheximide. Cells expressing the Na,K-ATPase $\alpha$ and $\beta$ polypeptides were fused as in Fig. 7 except that the after-fusion chase occurred in the absence of cycloheximide. Syncytia were fixed and analyzed by double labeled confocal microscopy. The $\alpha$ subunit distribution is shown in the bottom left. The $\beta$ subunit distribution is shown in the upper right.

Alternatively, assembly outside the ER may be unique to a set of proteins which include the Na,K-ATPase. Musil and Goodenough (1993) have shown that the integral membrane protein, connexin 43, can oligomerize outside the ER. Assembly of these proteins may not require the assistance of specific ER resident chaperone proteins or other factors in the ER environment necessary for assembly of other multimeric proteins. Since unassembled proteins usually are retained in the ER in vivo, it has been impossible to determine if residence in the ER is an absolute requirement for assembly. However, using the baculovirus-directed fusion system and hybrid ATPase molecules, we have found an opportunity to address this question.

The Na,K-ATPase belongs to a family of P type ATPases, the majority of which are multimeric, and whose minimum functional unit is a single polypeptide with homology to the Na,K-ATPase $\alpha$ subunit. These monomeric ATPases (e.g., H-ATPase, Ca-ATPase) couple ATP hydrolysis with unidirectional ion flux. Interestingly, it is only the P type ATPases that countertransport ions, the Na,K-ATPase (Na,K) and the H,K-ATPase (H,K), that require an associated $\beta$ subunit for activity (reviewed in Mercer, 1993). Moreover, it has been demonstrated in yeast (Eakle et al., 1992) and in *Xenopus* oocytes (Horrisberger et al., 1991) that the H,K $\beta$ subunit assembles with the Na,K $\alpha$ subunit and that these complexes are functional Na,K-ATPase molecules as defined by the

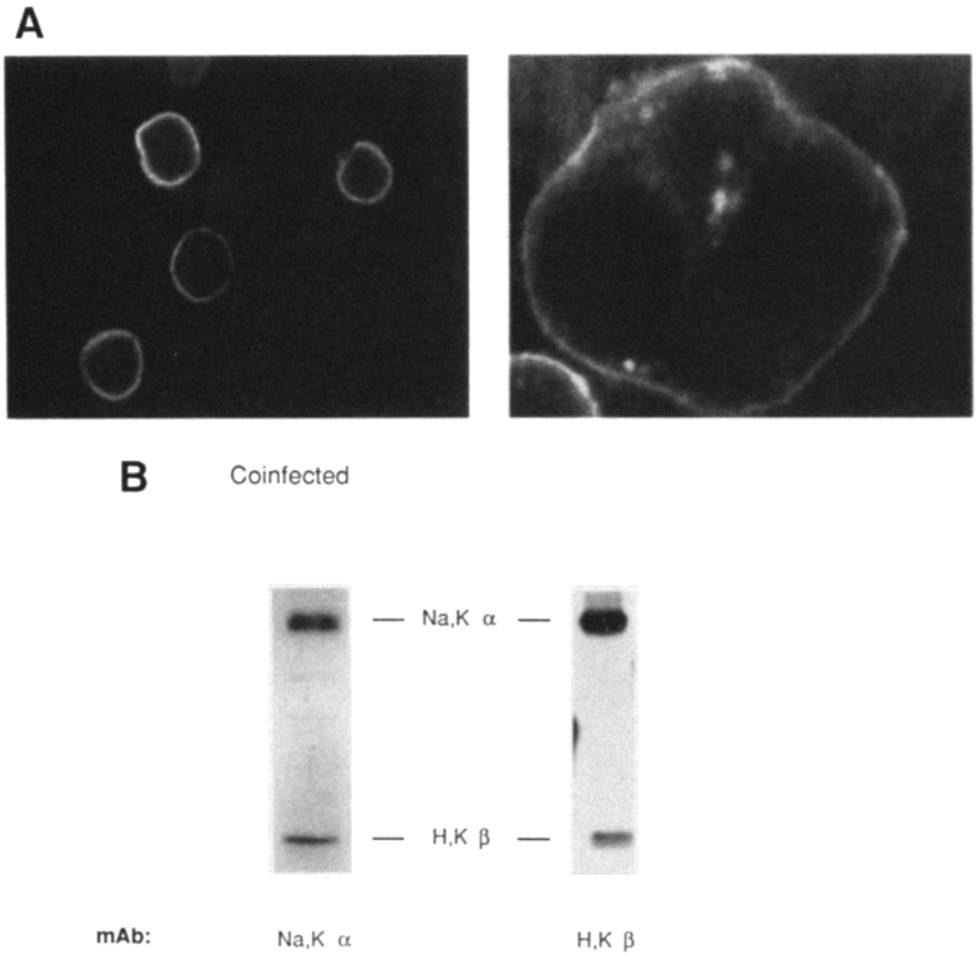



*Figure 9.* The H,K-ATPase $\beta$ subunit is targeted to the plasma membrane when expressed alone and associates with the Na,K-ATPase $\alpha$ subunit in the ER of coinfected cells. (*A*) The distribution of H,K-ATPase $\beta$ polypeptides in individually infected *Sf*-9 cells before (*left*) and after fusion (*right*) was analyzed by confocal microscopy. (*B*) Cells coinfected with the Na,K $\alpha$ and H,K $\beta$ were metabolically labeled for 20 min and chased for 30 min. Cell lysates were immunoprecipitated with either a mAb specific for the Na,K $\alpha$ or the H,K $\beta$ subunit. Both immunoprecipitates were digested with glycan F.

ability to bind ouabain and mediate a ouabain-sensitive potassium flux. Expression of the Na,K $\alpha$ with the H,K $\beta$ using recombinant baculoviruses indicates that these subunits are capable of forming stable and specific associations in insect cells (Koster et al., 1993). The two $\beta$ subunits share over 40% amino acid homology, are approximately the same size and are predicted to have the same membrane topography (Reuben et al., 1990). Although the H,K $\beta$ subunit can coexist with the Na,K subunits, it is not known if functional Na,K $\alpha$/H,K $\beta$ hybrid ATPases are formed in vivo. Using a H,K $\beta$ recombinant baculovirus and the cell fusion assay we wanted to test the ability of the H,K $\beta$ subunit to assemble with the Na,K $\alpha$ subunit at the plasma membrane of fused *Sf*-9 cells.

Similar to the Na,K $\beta$ polypeptides, the unassociated H,K $\beta$ polypeptides are targeted to the plasma membrane (Fig. 9 *A*), and also sediment to a single peak on sucrose gradients (not shown) when expressed in baculovirus-infected *Sf*-9 cells. The unassociated H,K $\beta$ subunit is also targeted to the plasma membrane in transfected mammalian cells (Gottardi and Caplan, 1993). Syncytia formation is the same as for the Na,K $\alpha$ and $\beta$ polypeptides. H,K $\beta$ polypeptides at the plasma membrane redistribute around the syncytium upon fusion with little intracellular localization of subunit. If cells are coinfected with viruses expressing the H,K-ATPase $\beta$ subunit and the Na,K-ATPase $\alpha$ subunit, the polypeptides assemble and can be coimmunoprecipitated with either the anti-Na,K-ATPase $\alpha$, or anti-H,K $\beta$ antibodies (Fig. 9 *B*). Syncytia formed between cells individually infected with the Na,K $\alpha$ subunit virus and the H,K $\beta$ subunit virus are identical to those seen with both Na,K-ATPase viruses (Fig. 10 *A*). The expressed Na,K $\alpha$ (*left*) and H,K-ATPase $\beta$ (*right*) polypeptides are targeted to the plasma membrane and upon fusion are redistributed around the perimeter of the syncytium. Intracellular labeling is slight, and the only location where the distribution of the two polypeptides overlap is at the plasma membrane. To ascertain if these two polypeptides can assemble outside the ER, H,K $\beta$-infected and Na,K $\alpha$-infected cells were plated together, the proteins were metabolically labeled and the cells fused as in the previous experiments.

Immunoprecipitation from adjacent nonfused and fused cells with the anti-Na,K $\alpha$ antibody are shown in Fig. 10 *B*. In contrast to the Na,K $\beta$ subunit, the H,K $\beta$ subunit did not coimmunoprecipitate with the Na,K $\alpha$ subunit from the fused cells. This was not due to differences in the expression levels of H,K $\beta$-infected cells compared to Na,K $\beta$-infected cells, nor was incomplete or selective syncytium formation observed. It appears that the Na,K $\alpha$ subunit can assemble with its complementary Na,K $\beta$ subunit both in the ER and at the plasma membrane, but can only assemble with the H,K $\beta$ subunit in the ER.

To further examine this result, cells infected with the Na,K $\alpha$ virus were fused to cells coinfected with both the Na,K

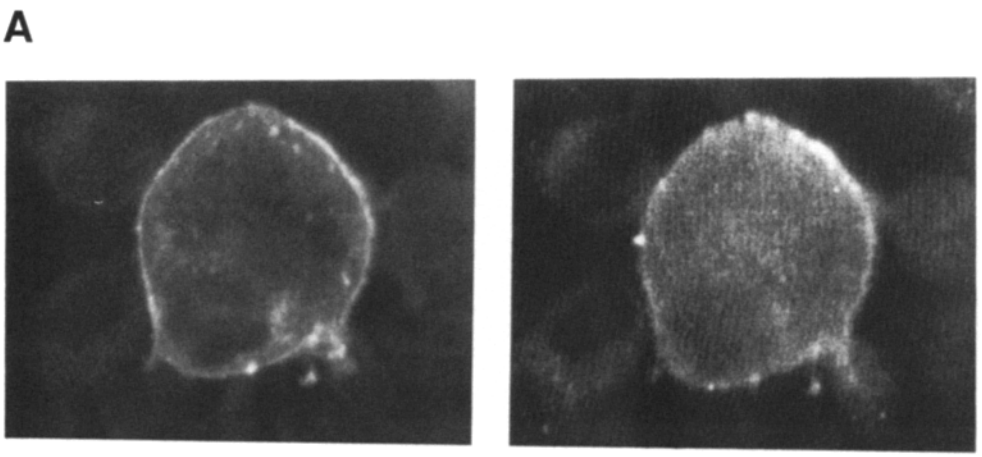



*Figure 10.* The hybrid ATPase does not assemble at the plasma membrane of Na,K $\alpha$/H,K $\beta$ syncytia. (*A*) The distribution of the Na,K $\alpha$ (*left*) and H,K $\beta$ (*right*) polypeptides in a four cell Na,K $\alpha$/H,K $\beta$ syncytium was analyzed by double-labeled confocal microscopy. (*B*) Immunoprecipitations with the Na,K $\alpha$ mAb from metabolically labeled Na,K $\alpha$ and H,K $\beta$ infected cells plated together (nonfused) or plated and fused (*fused*).

and H,K β viruses (Fig. 11). The top panels show double-labeled confocal analysis of a representative field of the Na,K β/H,K β coinfected cells. Over 90% of the cells are expressing both the Na,K β (*left*) and the H,K β (*right*) polypeptides. Aliquots from this same population of coinfected cells were seeded with Na,K α-infected cells and assayed for fusion-dependent assembly as previously described. Immunoprecipitates using an anti-Na,K α antiserum demonstrate that within these syncytia, the Na,K α selectively associates with the Na,K β subunit (Fig. 11 *B*). Aliquots of the detergent-solubilized syncytia were also immunoprecipitated with an anti-H,K β antibody that can coimmunoprecipitate the Na,K α from coinfected cells (Fig. 10 *B*). This antibody precipitates a large amount of H,K β polypeptides from the fused cells, however, Na,K α polypeptides are not detected.

These results demonstrate that the Na,K α subunit selectively associates with its complementary Na,K β subunit at the plasma membrane. This is in contrast to what is observed when cells are coinfected with the Na,K α, Na,K β and H,K β viruses. In this case, a mixture of Na,K α/Na,K β and Na,K α/H,K β heterodimers are detected, and this remains true even if one of the β subunits is expressed in excess over the other (Fig. 11 *C*). Thus, in the ER it appears that association between these three polypeptides is random, while at the plasma membrane the association is specific between subunits of the Na,K-ATPase. This demonstrates that assembly of some multisubunit proteins is restricted to the ER and suggests that ability of the Na,K-ATPase to assemble at the plasma membrane may be physiologically relevant.

## *Discussion*

Previous studies of Na,K-ATPase biosynthesis suggest that assembly of the α and β polypeptides in the ER occurs soon after synthesis. In chick primary neurons, αβ heterodimers

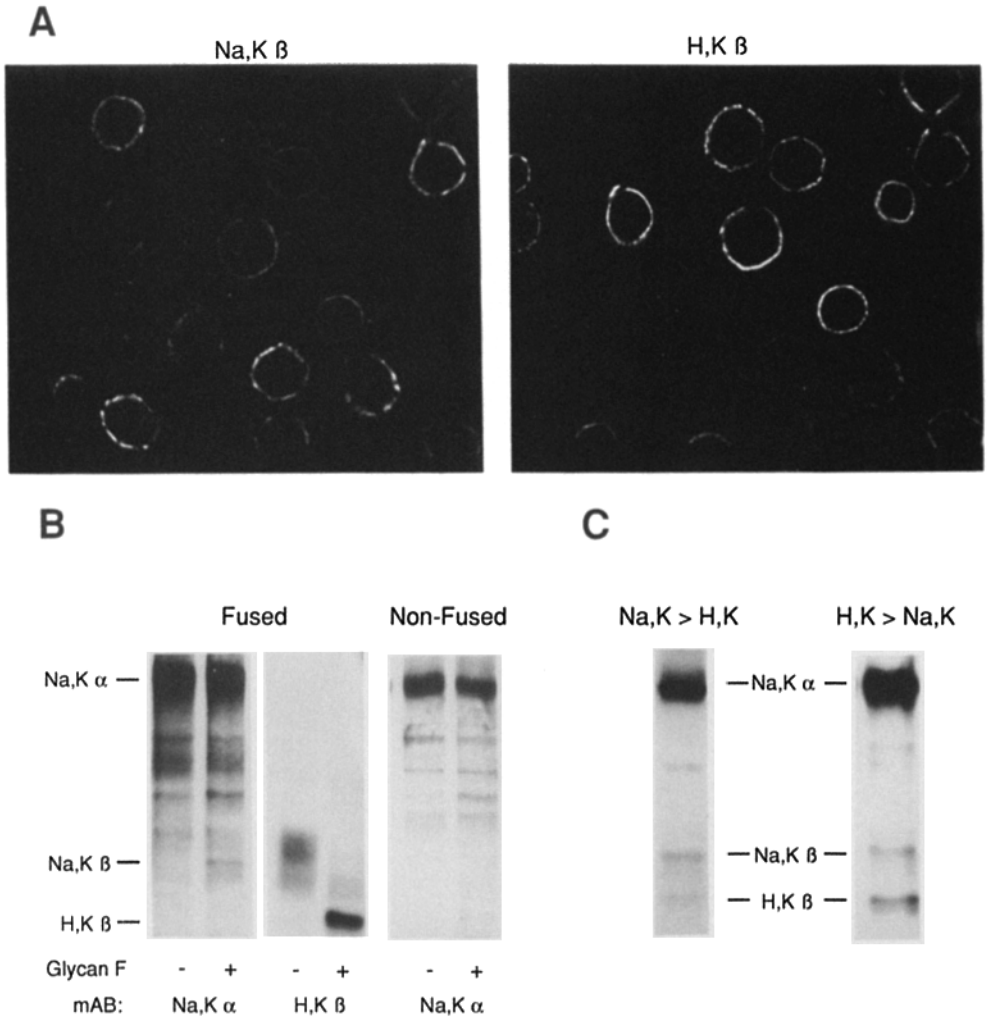



*Figure 11.* The Na,K α subunit selectively associates with the Na,K β subunit at the plasma membrane but not in the ER. Syncytia were formed between cells expressing the Na,K α subunit and cells co-expressing both the Na,K β and H,K β subunits. (*A*) Double-labeled confocal analysis of the coinfected cells show that the majority of cells are expressing both the Na,K β polypeptides (*left*), and the H,K β polypeptides (*right*). (*B*) Immunoprecipitations with the Na,K α mAb from fused cell lysates demonstrate that the Na,K α subunit selectively assembles with the Na,K β subunit at the plasma membrane. Immunoprecipitates with an anti-H,K β mAb precipitate large amounts of H,K β polypeptides but do not coprecipitate the Na,K α subunit. (*C*) Immunoprecipitations with the Na,K α mAb from cells coinfected with all three baculoviruses demonstrate that assembly of the Na,K α with the Na,K β and H,K β is random in the ER. If the Na,K β subunit is expressed in a greater amount than the H,K β subunit (Na,K > H,K), formation of the Na,K-ATPase is favored. In contrast, if the H,K β subunit is expressed in a greater amount than the Na,K β subunit (H,K > Na,K), formation of the hybrid ATPase is favored. Both immunoprecipitates were digested with glycan F.

are detected while the β subunit is still in an endo H–sensitive, core glycosylated state (Tankun and Fambrough, 1986). Furthermore, in MDCK cells, ATP-dependent ouabain binding of the αβ heterodimer, is detected within 10 min of synthesis (Caplan et al., 1990). Studies using *Xenopus* oocytes also demonstrate that the usual site of Na,K-ATPase assembly is the ER. In the *Xenopus* oocyte, the α subunit is normally overexpressed relative to the β subunit. These excess α polypeptides remain in the ER and assemble with β polypeptides as they become available (Geering, 1991). Alternatively, when β mRNA is injected into *Xenopus* oocytes to artificially create the opposite situation, excess β polypeptides remain in the ER where they assemble with the newly synthesized α polypeptides (Noguchi et al., 1990). These experiments demonstrate that excess unassembled α and β subunits remain in the ER and are able to oligomerize long after synthesis. Thus, assembly of the Na,K-ATPase appears to be similar to oligomerization of other plasma membrane proteins in that individual subunits remain in the ER until they are properly assembled, effectively limiting assembly to the ER (reviewed in Hurtley and Helenius, 1989).

These results are in contrast to the processing of Na,K-ATPase polypeptides in baculovirus-infected *Sf*-9 cells. In these cells both α and β subunits are independently targeted to the plasma membrane. The delivery of unassembled Na,K-ATPase subunits to the plasma membrane is not a consequence of the infected *Sf*-9 cells failure to recognize and retain unassembled or improperly assembled proteins. As shown for several membrane and secreted proteins, infected Sf-9 cells correctly assemble and deliver multimeric proteins to their appropriate destination (Birnir et al., 1992; DeTomaso et al., 1993*b*; Klaasen et al., 1993). The cause for the difference in the processing of the Na,K-ATPase in the vertebrate and invertebrate cells is unknown. However, baculovirus-induced expression of the Na,K-ATPase subunits provides the unique opportunity to study the assembly of the enzyme. The initial aim of this study was to determine if unassembled Na,K-ATPase subunits retain the ability to associate and assemble in the *Sf*-9 cell plasma membrane, or if assembly is limited to the ER. For the Na,K-ATPase, it appears that assembly is not an ER-restricted process. When the plasma membranes of α- and β-infected *Sf*-9 cells are fused, the α and β subunits of the Na,K-ATPase can associate and assemble into functional enzyme. This assembly is not a result of the random collision and nonspecific assembly of the subunits. When cells expressing the Na,K α subunit are fused with cells expressing the H,K β subunit, the subunits do not assemble. However, the hybrid ATPase consisting of the Na,K α and H,K β subunits can assemble in the ER of coinfected cells. Thus the assembly of the Na,K-ATPase at the plasma membrane is a highly specific process that occurs only between the Na,K subunits and not the cognate H,K subunit.

As in any specific intermolecular association, oligomerization requires individual proteins to present unique domains that recognize and associate with each other. So once the H,K β polypeptide has folded and is in a stable conformation, what would prevent it from assembling with the complementary Na,K α subunit? The inability of the Na,K α and H,K β to assemble at the plasma membrane suggests that assembly requires ER resident proteins, and/or the distinctive physical properties of the ER. Previous studies of oligomeric proteins demonstrate that assembly is a process that includes conformational changes of the individual subunits. For example, influenza hemagglutinin consists of one 74-kD precursor (HAO) that assembles into a homotrimer. There are several antibodies that only recognize HAO when it is a trimer (Copeland et al., 1986), demonstrating that new epitopes appear as a result of assembly. Also, assembly of the individual subunits of larger multisubunit proteins (e.g., acetylcholine receptor, T cell receptor) is often sequential, through a series of defined partial intermediates (Green and Claudio, 1993; Gu et al., 1991; Blount et al., 1990; Klausner et al., 1991). Thus, the new domains that recognize polypeptides that add on later, must form on the early intermediates. Finally, as assayed by changes in their proteolytic sensitivity, a number of proteins have been shown to undergo conformational changes upon assembly. This has been shown for the Na,K-ATPase α subunit. Assembly with either the Na,K or H,K β subunits transforms α polypeptides from a trypsin sensitive to resistant form (Geering et al., 1986; Horrisberger et al., 1991). This requirement for conformational changes may restrict assembly of some proteins to the ER. For instance, resident ER chaperone proteins (e.g., HSP 94, Bip) may assist in the refolding of the individual subunits during assembly just as they are thought to aid in the initial folding of nascent polypeptides. In this case, assembly of the hybrid ATPase at the plasma membrane would be analogous to the in vitro folding of a denatured polypeptide; without assistance it is a slow and inefficient process (Gething and Sambrook, 1992). This assisted assembly is in contrast to assembly of the viral VSV G (Machamer et al., 1990), and SV5 HN proteins (Ng et al., 1989), as well as the acetylcholine receptor α subunit (Forsayeth et al., 1992; Blount and Merlie, 1991). It has been suggested that prior to oligomerization, Bip disassociates from these proteins after they have acquired a stable conformation. However, if assembly of these proteins involves subtle shifts from one conformation to another, the requisite association with the chaperone may be weak and not detected. In any case, the finding that the hybrid ATPase can only assemble in the ER suggests that for some oligomeric proteins, assembly is not a simple matter explained by diffusion and collision of the polypeptides in the membrane.

Assembly of some polypeptides, besides requiring chaperones, may also be sensitive to the physical properties of the membrane, and in this manner be restricted to the ER. For instance, the plasma membrane of eucaryotic cells contains a higher percentage of sphingolipids, glycolipids, and cholesterol compared to the ER bilayer. These differences in composition can greatly affect the properties of the bilayer. In particular, cholesterol is known to both thicken, and greatly increase the mechanical stability of lipid bilayers (reviewed in Bretscher and Munro, 1993). Integral membrane proteins have extensive electrostatic and hydrophobic interactions with the surrounding lipids, and the ability of two proteins to closely associate or shift conformations during assembly may be inhibited in a different membrane environment. Moreover, the ER has high concentrations of calcium (Baumann et al., 1991) and an oxidizing redox potential (Hwang et al., 1992) not present at the plasma membrane. Thus the assembly of the hybrid ATPase may be limited to the ER because of the necessity for the ER membrane environment.

The assembly of functional Na,K-ATPase at the plasma membrane raises some important questions concerning the mechanisms of assembly of multimeric membrane proteins. For example, in the insect cell, how do unassembled Na,K-ATPase subunits escape the ER retention mechanism? Generally unassembled proteins are retained until they are either assembled or degraded (Hurtley and Helenius, 1989). However, it is clear that some of the subunits of the P type multimeric ATPases can exit the ER without their complementary subunits (DeTomaso et al., 1993*a*; Gottardi and Caplan, 1993; Koster et al., 1993). It is possible that these proteins belong to a distinct group of multimeric membrane proteins whose individual subunits are recognized by the ER as being monomeric. Thus, although these proteins can assemble with their corresponding subunits, they can also exist as independent membrane proteins. The majority of ATPases belonging to the P type transporters are monomeric, and it is possible that the multimeric transporters have retained this characteristic. Alternatively it could be that unless there are specific factors that retain a protein in the ER (i.e., aggregation, degradation, ER retention signals, binding to ER resident proteins), assembled or unassembled membrane proteins will normally exit the ER. Thus, because the unassembled Na,K-ATPase subunits can exist in an unaggregated, stable conformation they exit the ER unassociated. More importantly, once the Na,K-ATPase subunits have left the ER they retain the ability to assemble into a functional enzyme. Moreover, in contrast to the promiscuity of the Na,K-ATPase $\alpha$ subunit in the ER, the assembly at the plasma membrane is specific to only the Na,K-ATPase subunits. This specificity suggests that the post-ER assembly of the Na,K-ATPase may be physiologically relevant and function as a novel mechanism for the regulation of activity.

Although the exact mechanisms are unknown, it has been well established that catecholamines, insulin, and prolonged muscle activity stimulate the Na,K-ATPase in a time period too short to be accounted for by new synthesis (Clausen and Tobin, 1989). Thus, Na,K-ATPase activity may be regulated by assembly of the enzyme outside the ER. Interestingly, there is evidence in mammalian cells that unassociated Na,K-ATPase $\alpha$ and $\beta$ subunits exist outside the ER. A $\beta$-specific monoclonal antibody reacts with a polypeptide in rat distal colon that does not colocalize with the $\alpha$ subunit (Marxer et al., 1989), and in cultured kidney cells there is a pool of unassociated $\beta$ subunits in basolaterally enriched membranes (Mircheff et al., 1990). Furthermore, Hundal et al. (1992) suggest that in rodent muscle, insulin-stimulated Na,K-ATPase activity is the result of translocation of $\alpha$ and $\beta$ subunits from different intracellular compartments to the plasma membrane. The Na,K-ATPase maintains the electrochemical gradients fundamental to cellular physiology, and the ability to specifically assemble outside the ER may provide a new mechanism for the regulation of ion transport.

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