

# Conversion of a Class II Integral Membrane Protein Into a Soluble and Efficiently Secreted Protein: Multiple Intracellular and Extracellular Oligomeric and Conformational Forms

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**Abstract.** The NH<sub>2</sub> terminus of the F<sub>1</sub> subunit of the paramyxovirus SV5 fusion protein (fusion related external domain; FRED) is a hydrophobic domain that is implicated as being involved in mediating membrane fusion. We have examined the ability of the FRED to function as a combined signal/anchor domain by substituting it for the natural NH<sub>2</sub>-terminal signal/anchor domain of a model type II integral membrane protein: the influenza virus neuraminidase (NA) protein. The hybrid protein (NAF) was expressed in eukaryotic cells. The FRED was shown to act as a signal sequence, targeting NAF to the lumen of the ER, by the fact that NAF acquired N-linked carbohydrate chains. Alkali fractionation of microsomes indicated that NAF is a soluble protein in the lumen of the ER, and the results of NH<sub>2</sub>-terminal sequence analysis showed that the FRED is cleaved at

a site predicted to be recognized by signal peptidase. NAF was found to be efficiently secreted ( $t_{1/2} \sim 90$  min) from the cell. By using a combination of sedimentation velocity centrifugation and immunoprecipitation assays using polyclonal and conformation-specific monoclonal antibodies it was found that extracellular NAF consisted of a mixture of monomers, disulfide-linked dimers, and tetramers. The majority of the extracellular NAF molecules were not reactive with the conformation-specific monoclonal antibodies, suggesting they were not folded in a native form and that only the NAF tetramers had matured to a native conformation such that they exhibited NA activity. The available data indicate that NAF is transported intracellularly in multiple oligomeric and conformational forms.

**I**NTEGRAL membrane proteins have been shown in general to contain two types of hydrophobic domain that are involved in targeting integral membrane proteins to the RER membrane, in initiating their translocation across the membrane, and in determining their topography in membranes. These are the cleavable hydrophobic NH<sub>2</sub>-terminal signal sequences that, after interaction with the signal recognition particle, initiate the membrane translocation process and the hydrophobic stop/transfer, or membrane anchorage domain, that functions to terminate translocation of the protein by forming a stable interaction with the lipid bilayer (reviewed in 3, 53). The majority of proteins that span the lipid bilayer once contain an NH<sub>2</sub>-terminal signal sequence, an NH<sub>2</sub>-terminal ectodomain, and a COOH-terminal stop/transfer sequence; these proteins have been called class I integral membrane proteins (14). However, a small class of proteins (class II) that span the membrane once contain an uncleaved hydrophobic segment near the NH<sub>2</sub>-terminus that acts as both an internal signal sequence directing integration into the membrane and as a domain that anchors the polypeptide. These proteins are oriented with an NH<sub>2</sub>-terminal domain in the cytoplasm and a COOH-terminal ec-

todomain. Some recent work has indicated that, except for the presence of a cleavage site, the cleaved signal of a class I integral membrane protein is structurally and functionally equivalent to the noncleaved signal sequences of class II integral membrane proteins (48).

The fusion protein (F protein)<sup>1</sup> of paramyxoviruses is an integral membrane protein that promotes the coalescence of membranes to cause cell fusion (19, 46). The F protein is synthesized in a precursor form (F<sub>0</sub>) which is activated by a host cell protease to form the biologically active disulfide-linked subunits F<sub>1</sub> and F<sub>2</sub> (46, 47). The NH<sub>2</sub> terminus of F<sub>1</sub> (fusion-related external domain; FRED) is considerably hydrophobic, and this domain is thought to be involved in mediating fusion. It has recently been shown that the SV5 FRED domain can form a stable interaction with membranes. When the FRED domain was removed from its normal internal position in the SV5 F protein and attached to the COOH termi-

1. *Abbreviations used in this paper:* Endo H, endo  $\beta$ -N acetylglucosaminidase H; F protein, fusion protein; FRED, fusion-related external domain; HA, hemagglutinin; HA-F, hemagglutinin-fusion protein hybrid; HN, hemagglutinin-neuraminidase; NA, neuraminidase; NAF, neuraminidase-fusion protein hybrid.

nus of a soluble reporter molecule (a soluble form of influenza virus hemagglutinin [HA]), the hybrid protein (HA-F) was converted to one with the properties of an integral membrane protein (37). It was, thus, of interest to determine if this position effect on the properties of the FRED domain would also apply when the FRED domain was placed at the NH<sub>2</sub> terminus of a protein. In yeast, it has been shown that the amino acid specificity of signal sequences must be low since a great many sequences can replace the signal sequence of yeast invertase (20). Thus, it would not be surprising if the SV5 FRED domain could act as a signal sequence. The more intriguing question was whether the FRED domain would stably anchor a protein in membranes by its NH<sub>2</sub> terminus (i.e., in an "upside-down" orientation as compared with HA-F) when it replaced the signal/anchor domain of a class II integral membrane protein. A possible complicating factor in the experiment is that the FRED domain contains a potential site for cleavage by signal peptidase as predicted by von Heijne's rule (52).

In this paper, we describe the construction and characterization of a hybrid protein (NAF) that contains the cytoplasmic tail and ectodomain of influenza virus neuraminidase (NA) with its signal/anchor domain replaced with the SV5 FRED domain. NA is a well characterized class II integral membrane glycoprotein that is a tetramer consisting of a globular box-shaped head (100 × 100 × 60 Å) folded into six antiparallel β sheets attached to a slender stalk (51). NA exists as a pair of disulfide-linked dimers and disulfide-linked tetramers, and these intermolecular bonds occur in the stalk region of the molecule (51). We provide data that indicate that the hybrid NAF molecule is a soluble protein that is secreted from cells. Previously, it has been proposed from studies using wild-type integral membrane proteins and mutant integral membrane proteins that do not fold correctly, including the influenza virus NA (5), that native folding and oligomerization is a prerequisite for transport out of the ER (16, 23, reviewed in 43). Here, we show that (a) the majority of intracellular and extracellular NAF molecules are monomers and dimers that are not in a native conformation and (b) only a small percentage of the secreted molecules form a tetramer consisting of a pair of disulfide-linked dimers, and only this molecular species is in a native form that is enzymatically active.

## Materials and Methods

### Cells

Monolayer cultures of the TC7 clone of CV1 cells were grown in DME supplemented with 10% FCS as described previously (38).

### Plasmid Construction

A full-length cDNA clone of the NA gene of influenza A/Tokyo/3/67 (H2N2) inserted at the Sal I site of pBR322 that was used previously (26) was kindly made available by Dr. Gillian Air (University of Alabama in Birmingham, Birmingham, AL). The NA-specific cDNA was excised from pBR322 by digestion with Sal I. The ends of the purified Sal I insert DNA were filled-in using T<sub>4</sub> DNA polymerase, Xho I linkers were added, and the DNA was digested first with FnuD II, which cuts the DNA at a unique site downstream of the region encoding the NH<sub>2</sub>-terminal signal/anchor, followed by digestion with Xho I to create a Xho I end suitable for ligation at the 3' end (mRNA sense) of the NA cDNA. The NA signal/anchor domain was replaced with the F protein FRED domain by using a Xho I-FnuD II synthetic DNA fragment. Four oligonucleotides (two sets of complemen-

tary pairs; see below), encoding the amino acids shown in Fig. 1., were synthesized by the Northwestern University Biotechnology Facility (Evanston, IL) on a DNA synthesizer (model 380B; Applied Biosystems, Inc., Foster City, CA) and were purified as described (37). Complementary oligonucleotides in H<sub>2</sub>O were incubated at 100°C for 3 min and annealed by incubation at 55°C for 10 min followed by cooling at room temperature for 10 min. Annealed oligonucleotides were ligated by incubation at 16°C for 16–18 h and then digested with FnuD II and Xho I. An ~20-fold molar excess of annealed and ligated oligonucleotides were ligated to the large NA FnuD II-Xho I insert DNA fragment. After heat inactivation of the T<sub>4</sub> DNA ligase, the ligated NA DNA was digested with Xho I and ligated to DNA from the SV-40 late region replacement vector pSV103 (39), which had been previously digested with Xho I and treated with bacterial alkaline phosphatase. The resulting plasmid was used to transform *Escherichia coli* strain DH1, and the nucleotide sequence of the first 235 nucleotides of the hybrid gene was verified to ensure that the reading frame had been maintained across the junction between the synthetic DNA fragment and the NA DNA. The oligos used were (a) 5'-TCG AGA ATG AAT CCA AAT CAA AAG TTT GCA GGG GTG GTG ATT GGA TTA GCT GCA TTA GGA GTA GCT ACT-3'; (b) 5'-GCC GCA CAG GTC ACT GCC GCA GTG GCA CTA GTA AAG GCA AAT GAA GGA TCC G-3'; (c) 5'-CTG TGC GGC AGT AGC TAC TCC TAA TGC AGC TAA TCC AAT CAC CAC CCC TGT AAA CTT TTG ATT TGG ATT CAT TC-3'; and (d) 5'-CGG ATC CTT CAT TTG CCT TTA CTA GTG CCA CTG CGG CAG TGA C-3'.

### Transfection, Radioisotopic Labeling of Polypeptides in Infected Cells, Immunoprecipitation, and SDS-PAGE

Transfection of recombinant DNA molecules into CV1 cells, preparation of virus stocks, and radioisotopic labeling of infected cell polypeptides at 40–48 h after infection were carried out as previously described (39), except that for metabolic labeling of infected cell proteins monolayers were incubated in methionine- and cysteine-free DME, and the proteins were labeled using Tran[<sup>35</sup>S] label (ICN Radiochemicals, Irvine, CA). When tunicamycin (1 μg/ml; Calbiochem-Behring Corp., La Jolla, CA) was used, it was added to cells 2 h before labeling and maintained during the labeling period. Immunoprecipitation was carried out as described previously (11, 24) using (a) a rabbit polyclonal serum to Tokyo/67 NA; (b) mAbs NT Tok 25/4, NT Tok 34/9, or NT Tok 67/7 made to A/Tokyo/67 NA; or (c) mAb Jap 113/2 made to A/Japan/305/57 NA (25, 27, 54). These antibodies were generously made available by Dr. Robert G. Webster (St Jude Children's Research Hospital, Memphis, TN). Samples were prepared for electrophoresis and analyzed by SDS-PAGE on 15% polyacrylamide gels as described (24). Quantitation of autoradiographs using a laser scanning densitometer was done as described (18).

### Endo β-N Acetylglucosaminidase H (Endo H) Digestions

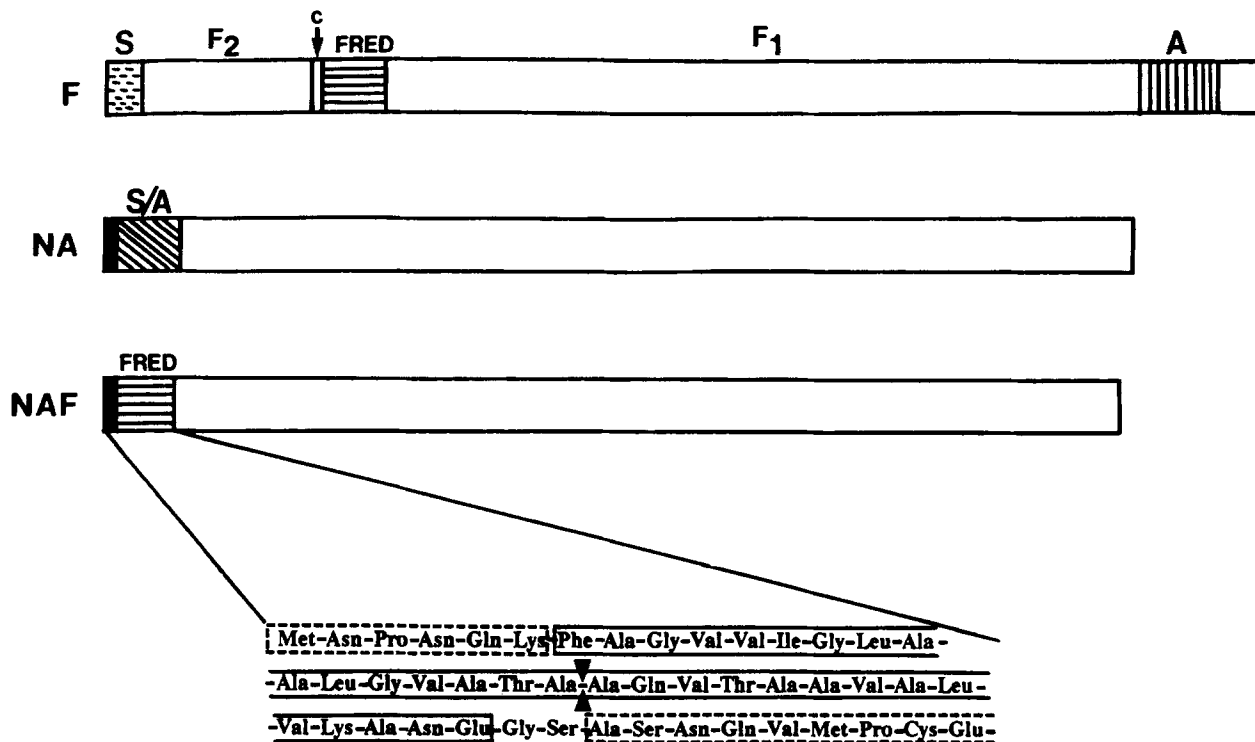
Endo H digestion of intracellular NAF protein was carried out as described previously (37). For Endo H digestion of secreted NAF protein, an equal volume of 2× RIPA buffer (24) was added to the supernatant medium from infected cells, and the samples were treated as above.

### Microsome Preparation and Protein Fractionation

Microsomes were prepared according to the method of Adams and Rose (1) as described previously (37). Alkali fractionation of microsomal proteins was performed as previously described (37).

### Sedimentation Velocity Centrifugation of NAF Protein on Sucrose Gradients

SV-40-NAF recombinant virus-infected CV1 cells were labeled for either 15 min or 1 h (depending on the experiment) with 200 μCi Tran[<sup>35</sup>S] label in 2 ml methionine- and cysteine-free DME/10-cm dish. After the labeling period, the medium was removed and replaced with 1 ml of DME containing 2 mM cold methionine and cysteine (chase medium), and incubation was continued for varying periods. The supernatant medium was centrifuged at 10,000 g for 5 min to remove any cellular debris, and an 0.1 vol of 10× lysis buffer (100 mM phosphate buffer, pH 7.2, 1 M NaCl, 10% Triton X-100) was added. The lysate (0.5 ml) was layered on top of a 10.5-ml 5–25% (wt/vol) sucrose gradient in 10 mM phosphate, pH 7.2, 100 mM NaCl, 0.1% Triton X-100 over a 0.75-ml 60% (wt/vol) sucrose cushion. The gradients were centrifuged for 16.75 h at 38,000 rpm at 20°C in an SW41



**Figure 1.** Schematic diagram illustrating the NAF hybrid protein. The SV5 F protein and the influenza virus NA are depicted at the top in schematic form. The F protein signal sequence (S) is illustrated as the dashed box, and the F protein stop/transfer membrane anchor domain (A) is illustrated by the vertically hatched box. The FRED domain is represented by the horizontally hatched box. The arrow marks the cleavage/activation (c) site of the F protein. The NA cytoplasmic tail of six amino acid residues is represented by the solid box, and the NA signal/anchor (S/A) domain is illustrated by a diagonally hatched box. The NAF recombinant molecule was constructed by annealing together four oligonucleotides and inserting them into a vector that expresses NA but from which the cytoplasmic tail and signal/anchor domain of NA had been deleted (see Materials and Methods). In the expanded insert to the NAF schematic diagram, the dashed rectangles show the amino acid residues encoded by NA (the six residues of the cytoplasmic tail and the beginning of the NA ectodomain), and the solid rectangle shows the 26 amino acids of the SV5 FRED domain followed by four residues of the F<sub>1</sub> subunit of the F protein. The Gly-Ser dipeptide located between FRED and the ectodomain is encoded by six nucleotides that were inserted into the construction because they contain a Bam HI restriction endonuclease site that facilitated identification of the correct recombinant DNA construction. The double arrowheads within the FRED domain indicate a site predicted by von Heijne's rule (52) that was a candidate site to be cleaved by signal peptidase.

rotor (Beckman Instruments, Inc., Fullerton, CA) before fractionation into 16 × 0.75-ml fractions from the bottom of the tube. For immunoprecipitation of gradient fractions, an equal volume of 2× RIPA buffer (24) was added to each fraction, and immunoprecipitation was performed as described above.

### NA Assays

NA activity assays were done as described previously (28). Since sucrose interferes with the colorimetric assay, all samples from sucrose gradients were dialyzed against 20 mM phosphate buffer, pH 6.0, overnight and concentrated in the dialysis bag against solid polyethylene glycol 8000. NAF NA activity was stable in 20 mM phosphate buffer, losing only 20% of its activity after 1 mo at 4°C.

### Direct Amino Acid Sequencing of the NH<sub>2</sub> Terminus of Secreted NAF

Monolayers of CV1 cells in 6-cm dishes were infected with a P1 stock of the SV40-NAF recombinant virus. At 40–48 h after infection, cell monolayers were labeled with 375 μCi/ml of [<sup>3</sup>H]valine (Amersham Corp., Arlington Heights, IL) in valine-free DME for 2 h. At the end of the labeling period, the radioactive medium was removed and replaced with 1 ml of DME containing 2 mM valine, and incubation was continued for an additional 2 h. The medium was collected and centrifuged at 45,000 rpm at 4°C in a TLA 100.2 rotor (Beckman Instruments, Inc.), an equal volume of 2×

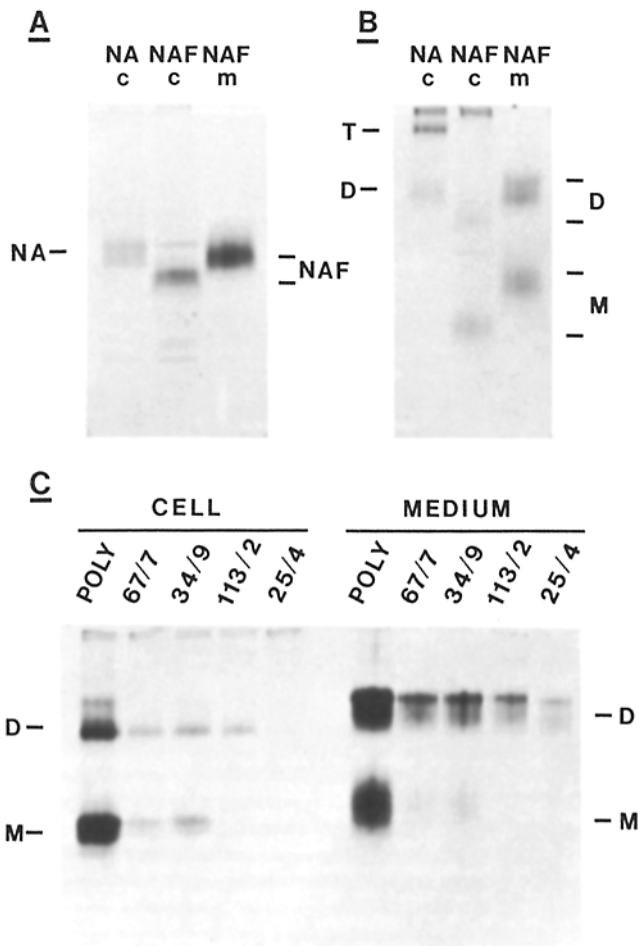
RIPA buffer (24) was added, and the samples were immunoprecipitated using rabbit polyclonal anti-Tokyo/67 NA serum as described above. After SDS-PAGE, the proteins were electroblotted to glass fiber paper, and NH<sub>2</sub>-terminal amino acid sequencing was done as previously described (40, 57) using a protein sequencer (Model 477A/120A; Applied Biosystems, Inc.).

## Results

### Rational and Construction of the NAF Molecule

We were interested in determining if the SV5 F protein FRED domain could function as an NH<sub>2</sub>-terminal signal/anchor domain of a type II integral membrane protein to both direct the translocation of a protein across the membrane of the ER and then, subsequently, to arrest translocation. The class II integral membrane protein reporter molecule that was chosen to investigate these properties of FRED was the influenza NA.

A DNA molecule was constructed, as described in Materials and Methods, such that a hybrid protein consisting of the cytoplasmic tail and ectodomain of NA with the SV5 FRED replacing the natural signal/anchor of NA would be



**Figure 2.** Secretion of NAF, intermolecular disulfide bond formation, and differing antibody reactivities. CV1 cells were infected with SV-40 recombinant viruses expressing NA or NAF, labeled with Tran<sup>[35S]</sup> label for 20 min, and then incubated in chase medium for 3 h (A and B). Both the medium (M) and the cells (C) were harvested and lysed in RIPA buffer. The labeled proteins were immunoprecipitated using the polyclonal Tokyo NA antisera and the polypeptides analyzed on SDS-PAGE under reducing (A) or nonreducing (B) conditions. Monomeric (M), dimeric (D), or tetrameric (T) forms of the polypeptides are indicated. (C) CV1 cells expressing NAF were labeled for 30 min with Tran<sup>[35S]</sup> label and either harvested immediately for analysis of intracellular NAF or incubated in chase medium for 3 h for analysis of secreted NAF. Proteins were immunoprecipitated with the rabbit polyclonal Tokyo NA antibody (lane POLY) or mAbs Tok 67/7, Tok 34/9, Jap 113/2, or Tok 25/4 (see Materials and Methods) and analyzed on SDS-PAGE under nonreducing conditions.

synthesized. In Fig. 1, the hybrid protein (NAF) is shown in schematic form in comparison with the wild-type NA and F proteins. Also shown in Fig. 1 is the sequence of the NH<sub>2</sub>-terminal 47 amino acids of NAF. Two residues (Gly and Ser) not found in either FRED or the NA ectodomain are present at the junction of FRED with NA since they are encoded by the nucleotides of a Bam HI site that was inserted to facilitate identification of the correct DNA construction. DNA fragments encoding either the NA or hybrid NAF proteins were inserted into the SV-40 late region replacement vector pSV103 (37), and the nucleotide sequence across the FRED-NA junction was verified.

### Expression and Glycosylation of NAF Protein in CV1 Cells

The SV-40 NA and NAF recombinant DNAs together with DNA from an SV-40 early region deletion mutant (42) were introduced into CV1 cells by means of DEAE-dextran-mediated transfection (32), and virus stocks were prepared. Monolayer cultures of CV1 cells were infected with first passage stocks of the SV-40 recombinant viruses, and infected cell proteins were labeled with Tran<sup>[35S]</sup> label at 40–48 h after infection. Cell lysates were prepared, immunoprecipitated with the polyclonal Tokyo NA serum, and analyzed by SDS-PAGE as described in Materials and Methods. As shown in Fig. 2 A, a protein with slightly increased electrophoretic mobility in comparison with NA was observed (Fig. 2 A, lanes NA c and NAF c).

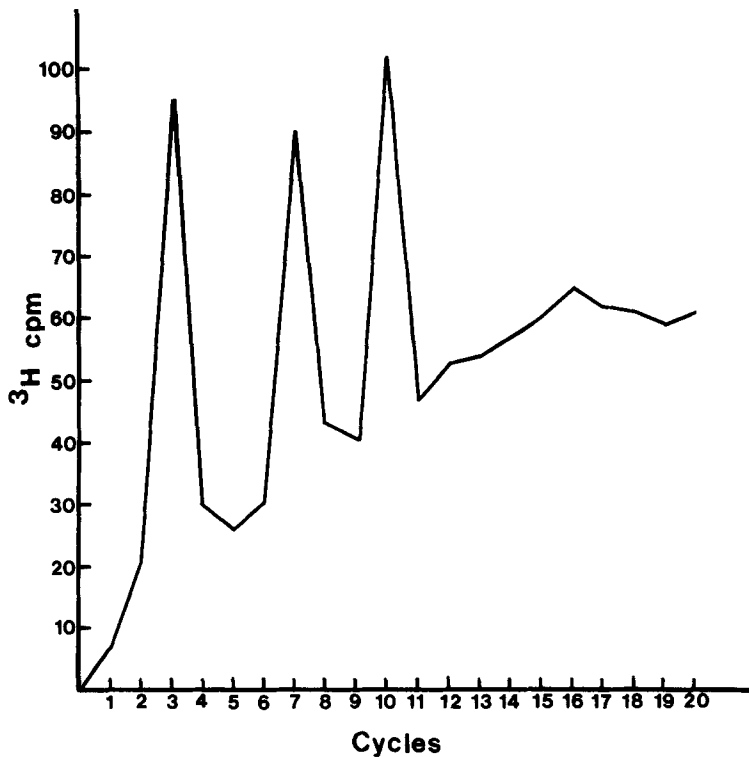
The addition of N-linked carbohydrate chains to proteins occurs in the lumen of the ER. Thus, glycosylation of NAF would be indicative of the SV5 FRED acting as a signal and directing the translocation of the NA ectodomain into the ER lumen. To investigate this possibility, NAF was synthesized in the presence of the inhibitor of N-linked glycosylation, tunicamycin (1 μg/ml). NAF synthesized in the presence of tunicamycin was observed to have a significantly increased electrophoretic mobility on SDS-PAGE as compared with NAF synthesized in the absence of the drug (data not shown). Thus, it was concluded that, as NAF possesses N-linked carbohydrate chains, the NAF ectodomain was translocated into the lumen of the ER.

To examine whether the FRED domain, in addition to acting as a signal sequence, had acted as a stop/transfer sequence, microsomes were isolated from cells expressing NAF and subjected to alkaline fractionation. At alkaline pH, integral membrane proteins remain associated with the lipid bilayer and are found in the pellet fraction, and soluble proteins and proteins peripherally associated with membranes are found in the supernatant (2, 17, 37, 49). NAF was found almost entirely in the supernatant fraction (data not shown), thus indicating that NAF is a soluble protein that is completely translocated into the lumen of the ER.

### Secretion of NAF, Intermolecular Disulfide Bond Formation, and Differing Antibody Reactivities

Since NAF was found to be a soluble protein in the lumen of the ER, it seemed possible that NAF would be secreted. CV1 cells expressing NAF were labeled for 20 min with Tran<sup>[35S]</sup> label and then incubated in chase medium for 3 h. The cell lysate and medium were immunoprecipitated with the polyclonal Tokyo NA antibody. As shown in Fig. 2 A, NAF was recovered from the medium (lane NAF m) as well as from cells (lane NAF c). Expression of NA is shown as a size marker (lane NA c), and no NA was found in the medium (data not shown). Secreted NAF had a slower electrophoretic mobility on SDS-PAGE than intracellular NAF; this is thought to be due to a difference in carbohydrate modification of the molecules. The nature of the minor bands seen in Fig. 2 A that coprecipitate with NA and NAF have not been investigated at this time.

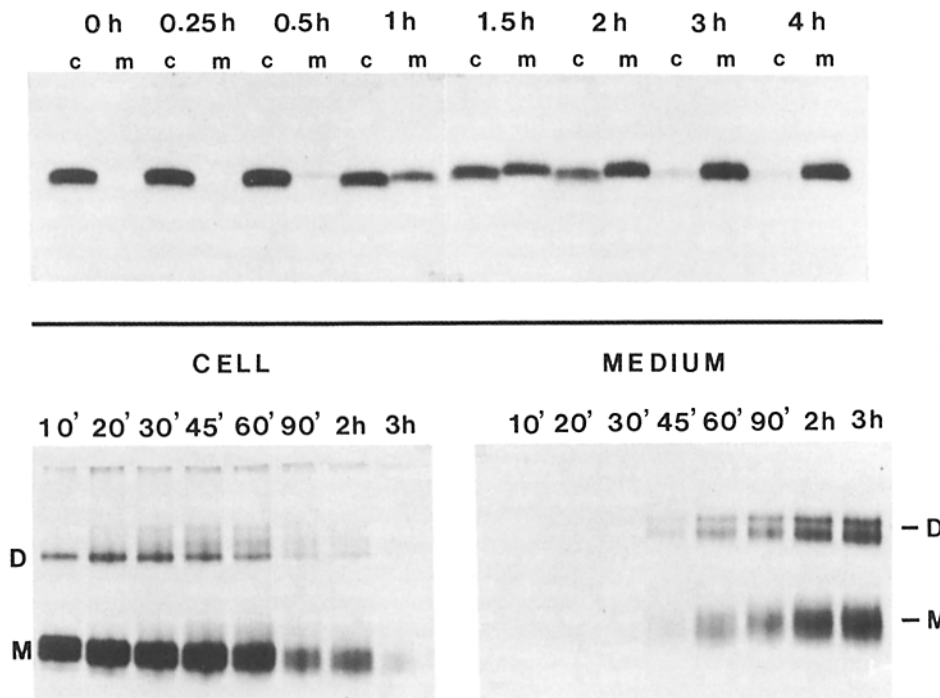
To examine for the formation of intermolecular disulfide bonds in NAF, intracellular and secreted NAF were subjected to electrophoresis under nonreducing conditions and compared with NA as a control. As shown in Fig. 2 B, NA



**Figure 3.** NH<sub>2</sub>-terminal sequence analysis of the NAF protein. CV1 cells were infected with the SV-40 recombinant virus expressing NAF and labeled with [<sup>3</sup>H]-valine, and the secreted NAF was immunoprecipitated from the medium with polyclonal Tokyo NA antiserum and analyzed on SDS-PAGE. Labeled protein was electroblotted onto activated glass fiber paper, and the region containing the NAF protein excised. Protein sequencing was done as described in Materials and Methods. The material from each cycle of Edman degradation was dried in a scintillation vial and counted in a model LS6800 scintillation counter (Beckman Instruments, Inc.). The radioactivity released from each of 20 cycles of Edman degradation is shown. The peaks of radioactivity at cycles 3, 7, and 10 correspond to valine at NAF residues 25, 29, and 32 (indicated by stars), and the arrow indicates that the deduced NH<sub>2</sub>-terminus of secreted NAF protein is the alanine at residue 23.

was found as a mixture of a pair of disulfide-linked dimers and disulfide-linked tetramers that migrated near the top of the gel. This confirms work reported previously (51). Intracellular NAF was predominantly found to migrate on SDS-

PAGE in a position compatible with it being nondisulfide linked (monomer) with a small amount forming a disulfide-linked dimer. In contrast, secreted NAF was found to exist as an approximately equal mixture of monomers and disul-



**Figure 4.** Kinetics of secretion of NAF. NAF recombinant virus-infected CV1 cells were labeled with Tran[<sup>35</sup>S] label for 10 min. The cells were then incubated for the times indicated in chase medium, and intracellular (c; CELL) and extracellular (m; MEDIUM) NAF was immunoprecipitated with the polyclonal Tokyo NA antibody. (Top) Samples analyzed on SDS-PAGE under reducing conditions; (bottom) Samples analyzed on SDS-PAGE under nonreducing conditions.

fide-linked dimers. The difference in amount of total radioactivity in the intracellular NAF species (lane *NAF c*) and the secreted NAF species (lane *NAF m*) reflects the 20-min labeling and 3-h chase period used since in this time a large amount of NAF was secreted (see below). The difference in stoichiometry between the intracellular monomers and dimers and those found extracellularly will be discussed further below.

mAbs that recognize conformational epitopes can be very useful reagents to examine the status of folding of a polypeptide (7, 8, 16, 33, 36, 56). To test the ability of different mAbs to react with the intracellular and extracellular NAF, CV1 cells expressing NAF were labeled for 30 min with Tran<sup>[35S]</sup> label, and the cells either harvested immediately or the medium was replaced with chase medium and the cells were incubated for 3 h before harvesting the medium. A large panel of mAbs to NA, shown previously to bind to different antigenic sites on the mature NA of purified influenza virions, were used to immunoprecipitate NAF using conditions of antibody excess. The precipitated NAF polypeptides were subjected to SDS-PAGE under nonreducing conditions. Examples illustrating the three different types of reactivity found are shown in Fig. 2 C. The rabbit polyclonal Tokyo NA sera was used to provide concentration and size marker lanes for the NAF monomeric and disulfide-linked species. mAbs Tok 67/7 and Tok 34/9 immunoprecipitated monomers and dimers in a similar ratio from cells, but these molecular species comprised only a subset of the total intracellular NAF molecules (c.f. lanes *POLY*, 67/7 and 34/9). These two mAbs also immunoprecipitated a subset of the extracellular NAF species that is found on gels as monomers and disulfide-linked dimers. In contrast, mAb Jap 113/2 only immunoprecipitated a subset of the intracellular and extracellular disulfide-linked molecules and none of the monomeric species. mAb Tok 25/4 (which was representative of 17 of 20 mAbs tested) only immunoprecipitated very small amounts of intracellular NAF but it did precipitate a readily detectable subset of the extracellular NAF dimeric species. The population of NAF molecules immunoprecipitated by mAb Tok 25/4 had a small but reproducible difference in electrophoretic mobility as compared with the species precipitated by the Jap 113/2 antibody, which may reflect either differences in intramolecular disulfide bond formation or differences in carbohydrate modification. In comparison with the data obtained with NAF, the three different mAbs could quantitatively immunoprecipitate influenza virus NA in amounts comparable with the polyclonal Tokyo NA sera (data not shown). It has been shown previously that the Jap 113/2 and Tok 25/4 mAbs bind to different sites on NA (26) and, whereas the Jap 113/2 antibody only slightly inhibits the NA activity of NA, the Tok 25/4 antibody completely inhibits NA activity (26; Paterson, R. G., and R. A. Lamb, unpublished observations). Thus, in aggregate, these data suggest that in the cell and medium species of NAF can be found that differ not only by the formation of intermolecular disulfide bonds but also by differing reactivities with specific antibodies, suggesting that intracellular and extracellular NAF exists in several folded states. In addition, the data indicate that the reactivity of the mAbs with the extracellular forms of NAF 3 h after the labeling period (particularly the dimer species) was much greater than that observed with the intracellular species after a 30-

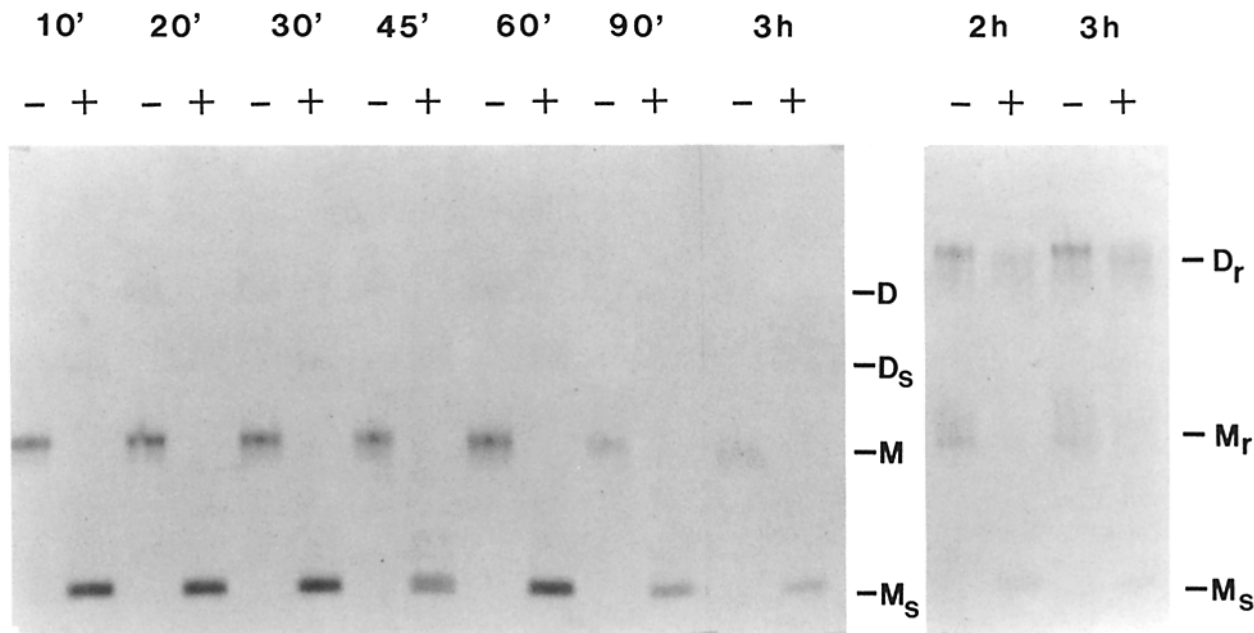
min labeling period, suggesting that the immunological reactivity of the NAF molecules matures with time.

### *NH<sub>2</sub>-terminal Sequencing of NAF Indicates Cleavage within the FRED Domain*

The finding that the FRED domain acted as a signal sequence to direct NAF to the ER membrane but then failed to act as a stop/transfer sequence suggested at least two possible explanations for the results obtained. Although the FRED domain could act as a stop/transfer sequence when it replaced such a domain at the COOH terminus of a type I integral membrane protein (37), when the FRED domain is placed at the NH<sub>2</sub> terminus of a type II integral membrane protein as in NAF, the hydrophobicity of the FRED domain may not be sufficient to halt transport of the growing NAF chain due to the driving force of protein translocation and/or chain synthesis. Alternatively, NAF could have become a soluble protein due to proteolytic cleavage of the FRED domain in the ER mediated either by signal peptidase or another ER proteolytic activity. To distinguish between these possibilities, the amino acid sequence of the NH<sub>2</sub> terminus of purified [<sup>3</sup>H]valine-labeled NAF was determined. As shown in Fig. 3, peaks of <sup>3</sup>H radioactivity were recovered after Edman degradation cycles 3, 7, and 10. The only place in the entire NAF sequence where valine is found with this periodicity is within the FRED domain, and, therefore, the mature NH<sub>2</sub> terminus of NAF is alanine at NAF residue 23. Thus, a proteolytic cleavage event had occurred in the hydrophobic FRED domain of NAF.

### *Rate of Secretion of NAF*

To determine the rate of secretion of NAF from cells, recombinant virus-infected cells were labeled with Tran<sup>[35S]</sup> label for 10 min and then incubated in chase medium for varying periods up to 4 h. Intracellular and secreted NAF were immunoprecipitated with polyclonal Tokyo NA antibody and analyzed on SDS-PAGE under reducing conditions. As shown in Fig. 4, *top*, >90% of NAF is secreted in 4 h of chase period, and the rate of NAF secretion has a *t*<sub>1/2</sub> of ~90 min, a rate that is very similar to that for transport of NA to the cell surface (5; Paterson, R. G., and R. A. Lamb, unpublished observations). Since virtually all the synthesized NAF molecules were secreted in 4 h, it indicates that secretion of NAF is efficient. Analysis of samples under non-reducing conditions from a similar experiment (Fig. 4, *bottom*) showed a more complex series of intracellular and extracellular NAF species. In the cell, immediately after the pulse label, the majority of NAF was monomeric. During the chase period, NAF exhibited a faster electrophoretic mobility that was presumably due to trimming of mannose residues from the core oligosaccharides, a process known to begin in the ER and continue in the Golgi apparatus (22). In addition to NAF monomers, some disulfide-linked NAF dimers were observed 10 min after the labeling period, and, within 20 min, two slower migrating NAF dimeric species were observed. These data did not demonstrate straightforward kinetics for the interconversion of the monomer to the dimer form of NAF nor did they show the interconversion of the faster migrating NAF dimer form to the slower migrating forms. However, with time the intracellular NAF monomers



**Figure 5.** Secreted NAF carbohydrate chains are resistant to digestion with Endo H. NAF recombinant virus-infected CVI cells were labeled with Tran<sup>[35S]</sup> label for 15 min and incubated in chase medium for the varying indicated times. Intracellular or secreted NAF molecules were immunoprecipitated with polyclonal Tokyo NA antiserum, incubated with (+) or without (-) Endo H as described in Materials and Methods, and analyzed on SDS-PAGE under nonreducing conditions. (*Left*) Intracellular NAF; (*right*) secreted NAF. *D* and *M*, disulfide-linked dimers and monomers, respectively; *D<sub>s</sub>* and *M<sub>s</sub>*, Endo H-sensitive dimers and monomers, respectively; *D<sub>r</sub>* and *M<sub>r</sub>*, Endo H-resistant dimers and monomers, respectively.

and all three dimer species were lost from the cell. In the medium, NAF monomer and disulfide-linked dimer species could be identified and they accumulated with time. Nearly equivalent amounts of extracellular NAF monomers and disulfide-linked dimers were found, and they were in a ratio different to that found for the intracellular NAF species. Thus, the simplest explanation for these data is that after formation of the interchain disulfide bond NAF dimers rapidly exit from the cell. In addition, these data indicate that formation of the disulfide bond is not obligatory for secretion of NAF from the cell. The reactivity pattern of the conformation-specific mAbs was investigated in a pulse label and chase protocol using the same time points as shown in Fig. 4. However, very little intracellular NAF was precipitated by the mAbs at any time, and reactivity for the mAbs did not change with time (data not shown). These data are compatible with the notion that after acquisition of reactivity for the mAbs NAF is rapidly secreted.

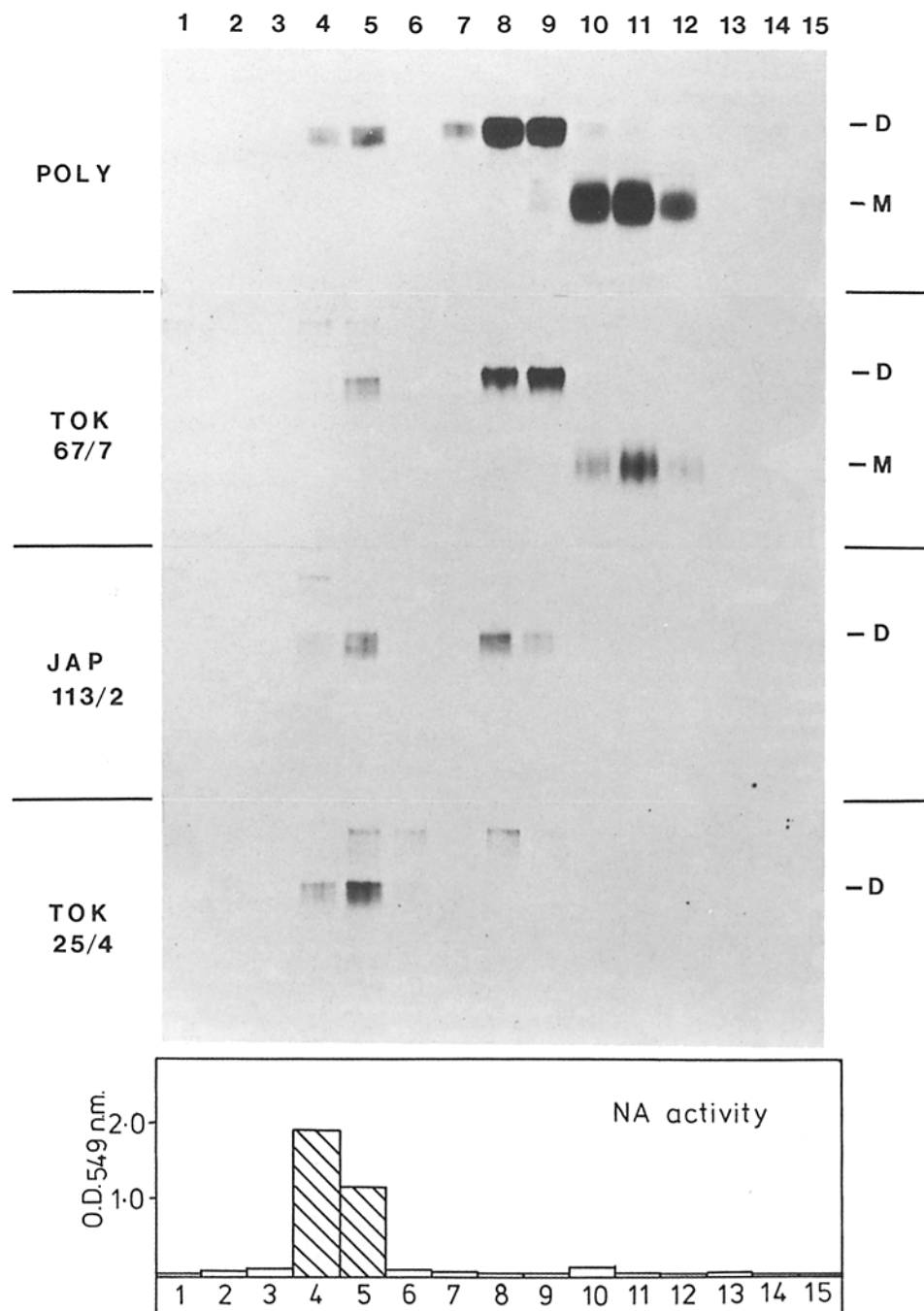
#### **Acquisition of Endo H-resistant Carbohydrate Chains on NAF**

To determine whether NAF acquired resistance to Endo H digestion, indicative of the conversion of carbohydrate chains from the simple to complex form in the *medial*-Golgi complex, recombinant virus-infected cells were labeled with Tran<sup>[35S]</sup> label for 15 min and incubated in chase medium for varying periods. Intracellular and secreted NAF were immunoprecipitated, incubated with or without Endo H, and analyzed on SDS-PAGE under nonreducing conditions (Fig. 5). The majority of intracellular NAF molecules were found

to be sensitive to Endo H digestion, and, as would be expected from the data shown in Fig. 4, they were found largely to consist of the NAF monomer species. In contrast, almost all the detectable extracellular NAF dimers and 65% of the extracellular NAF monomers were resistant to Endo H digestion. However, 35% of extracellular NAF monomers were Endo H sensitive. The simplest explanation of the observation that very small amounts of dimeric Endo H-resistant product are found in the cell whereas extracellular dimeric NAF is largely resistant to Endo H digestion is that, after modification of the NAF N-linked carbohydrate chains from the simple to complex type, the molecules are rapidly secreted. The finding that a proportion of the monomers were sensitive to Endo H digestion suggests that these monomers are passing through the Golgi apparatus in a form that is less accessible to the processing enzymes.

#### **Oligomeric Forms of the Extracellular NAF Protein**

To examine the oligomeric form of secreted NAF by a non-denaturing assay, radioactively labeled secreted NAF was fractionated by centrifugation on sucrose density gradients. Fractions were collected from the bottom of the gradients and immunoprecipitated with the four NA antisera that have different specificities, and the polypeptides were analyzed on SDS-PAGE under nonreducing conditions. Fractions were also assayed for NA enzyme activity. Three discrete NAF species were identified using the rabbit polyclonal Tokyo NA antibody (Fig. 6, *POLY*). Near the bottom of the gradient, in fractions 4 and 5, an NAF species was found that on gels

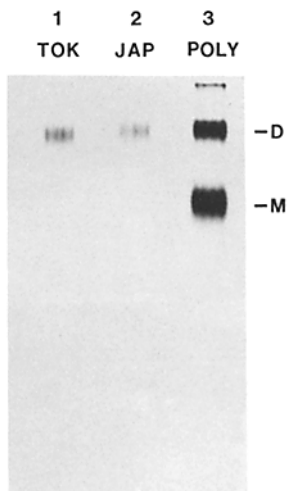


**Figure 6.** Analysis of oligomeric forms of extracellular NAF on sucrose density gradients and their differing antibody and enzymatic activities. NAF recombinant virus-infected CV1 cells were labeled with Tran<sup>[35S]</sup> label for 1 h and incubated in chase medium for 2 h. The NAF secreted into the medium was fractionated on sucrose gradients as described in Materials and Methods. Samples collected from the bottom of the gradient were immunoprecipitated with the polyclonal Tokyo NA antisera or the three conformation-specific mAbs Tok 67/7, Jap 113/2, or Tok 25/4. NA activity assays on sucrose density gradient fractions were performed as described in Materials and Methods. (Fraction 1) Bottom of the gradient; (fraction 15) top of the gradient. Polypeptides were analyzed on SDS-PAGE under non-reducing conditions. D and M indicate the approximate electrophoretic positions of the various disulfide-linked dimeric and monomeric species, respectively.

was a dimer, and this NAF species cosediments with NA (data not shown). Thus, this species of NAF is presumed to be a tetramer composed of a pair of disulfide-linked dimers. In the middle of the gradient, in fractions 7-9, an NAF species was found that on gels was also a disulfide-linked dimer and thus by its sedimentation characteristics is presumed to be an NAF dimer. Near the top of the gradient (fractions 10-12), an NAF species was found that on gels was a monomer, and, from its sedimentation characteristics, it seems reasonable to assume it is an NAF monomer. It should be noted that only disulfide-linked dimers or pairs of disulfide-linked dimers could be recovered from the NAF species sedimenting in the dimeric and tetrameric positions on the

gradient and that no nondisulfide-linked dimeric or tetrameric species were observed. The mAb Tok 67/7 immunoprecipitated the same three NAF species (Fig. 6, TOK 67/7) as the polyclonal Tokyo NA antibody. However, the ratio of the amount of NAF monomer as compared with oligomeric NAF precipitated by this mAb was different from that found with the polyclonal antibody. This suggests that the NAF monomer exists in more than one conformational form which can be distinguished by use of the two antisera. The mAb Jap 113/2 (Fig. 6, JAP 113/2) only recognized the NAF tetramer and a proportion of the dimer forms and did not precipitate any NAF monomer. The mAb Tok 25/4 (Fig. 6, TOK 25/4) only precipitated the NAF tetramer and not the dimers or





**Figure 7.** Sequential immunoprecipitations of secreted NAF for depletion of specific antibody-reactive forms. NAF recombinant virus-infected CV1 cells were labeled with Tran[<sup>35</sup>S] label for 1 h and incubated in chase medium for 2 h. The medium was incubated sequentially with two rounds of an excess of mAbs Tok 25/4 and Jap 113/2 and the rabbit polyclonal Tokyo NA antibody. Antibody at each stage was removed by addition of protein A-agarose beads. Polypeptides were analyzed by SDS-PAGE under non-reducing conditions. Only the material precipitated from the first rounds of immunoprecipitation are shown since the second rounds contained <5% of antibody-reactive NAF species.

monomers. Thus, these data suggest that the tetramers, dimers, and monomers of NAF have some epitopes that are shared and some that are oligomer specific and thus presumably the three forms also have different folded states. A further indication of a molecule approaching its native conformation is that it has enzymatic activity. Consequently, NAF species separated on sucrose gradients were assayed for NA activity. As shown in Fig. 6, *bottom*, the only species of NAF associated with NA activity was the tetrameric form. Approximately 90% of the enzymatic activity loaded onto the sucrose gradient was recovered in the tetramer-containing fractions, indicating that the centrifugation conditions had not inactivated biological activity. The mAb Tok 25/4, but not mAb Jap 113/2, has been shown previously to inhibit enzymatic activity (26). Addition of mAb Tok 25/4 to the gradient fractions before the NA assay completely eliminated all enzymatic activity (data not shown), which provides further evidence that the tetrameric form of NAF recognized by the mAb Tok 25/4 is the biologically active form. Unfortunately, because of the low amounts of NAF protein produced by the SV-40 recombinant vectors it was not possible to derive an enzyme-specific activity for NAF and to compare it with that for NA.

To exclude further the possibility that the NAF dimers and monomers only appeared on sucrose gradients due to the disruption of tetramers under the centrifugation conditions, sequential rounds of depletion immunoprecipitation were performed on secreted NAF using antibodies of differing reactivities. NAF was first precipitated in two sequential immunoprecipitations with mAb Tok 25/4 that is specific for the enzymatically active and tetrameric NAF. The supernatant, depleted of NAF molecules reactive with this antibody, was then immunoprecipitated in two sequential immunoprecipitations with mAb Jap 113/2. Lastly, the supernatant depleted of NAF species reactive with the mAbs was immunoprecipitated with the polyclonal Tokyo NA antibody. When the polypeptides from the sequential reactions were analyzed under nonreducing conditions on SDS-PAGE (Fig. 7), the antibodies were found to precipitate the same species and in

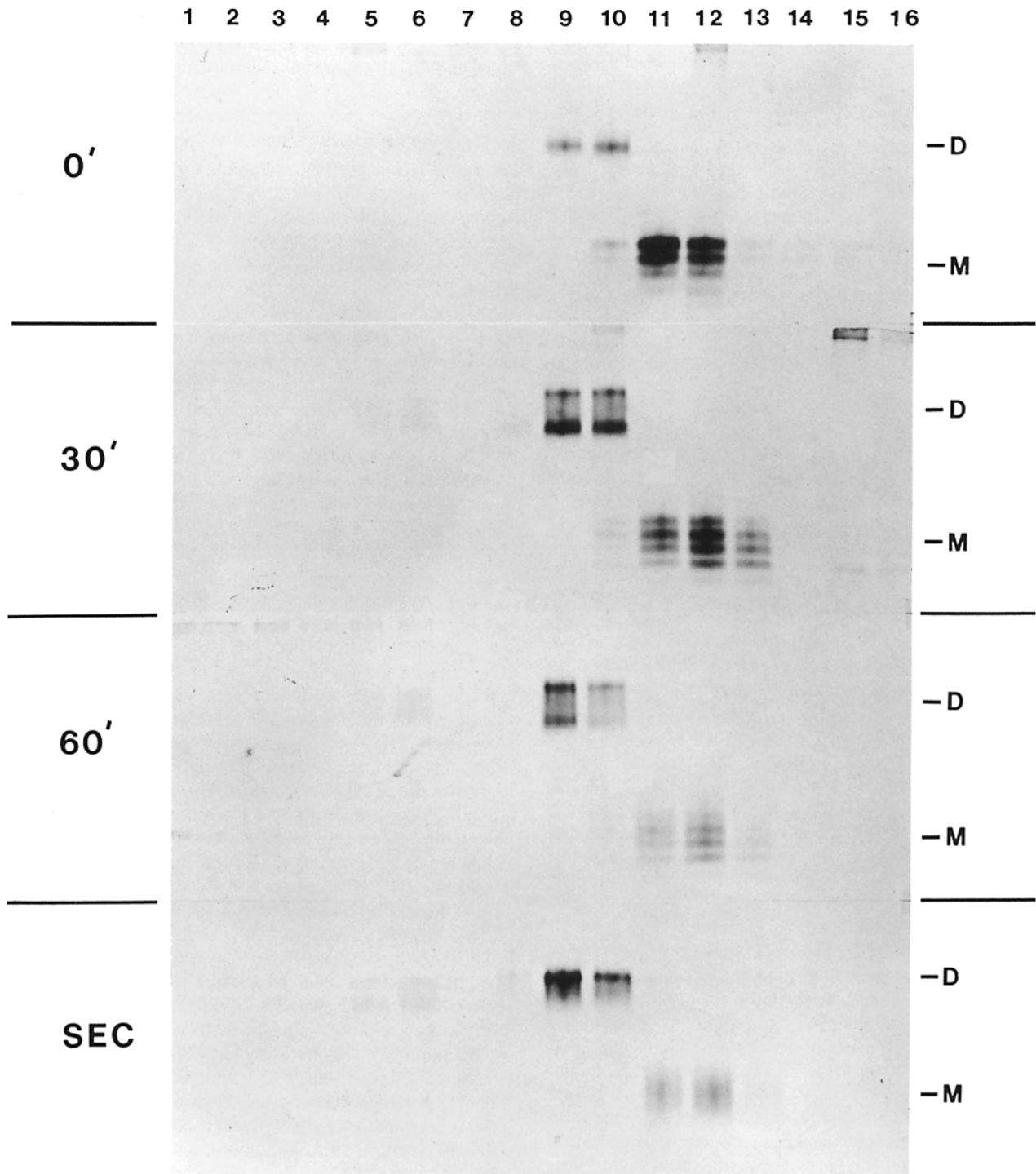
similar stoichiometry as found in Fig. 2, which strongly suggests that NAF exists in the medium in different conformational and oligomeric forms before being subject to centrifugation.

### *The Oligomeric Forms of Intracellular NAF*

For proteins transported through the exocytotic pathway, including influenza virus NA, it has been found that native folding and oligomerization is a prerequisite for transport out of the ER (5, 16, 23, 43). Although the majority of intracellular and extracellular NAF molecules are monomers and dimers that do not react with the Tok 25/4 oligomer-specific mAb, the data described above does not provide information about the oligomeric form of the molecule during intracellular transport. It could be argued that the antigenically immature extracellular NAF monomers were not secreted in that form but instead formed an antigenically mature tetramer of nondisulfide-linked molecules for intracellular transport and because of the tetramer's rapid secretion immediately after its formation it could not be detected using the Tok 25/4 oligomer-specific mAb. On secretion, this hypothetical species would have to dissociate and undergo conformational changes to yield the antigenically immature monomers. If an mAb were available that only recognized antigenically immature NAF monomers, it would be a very useful reagent to study this problem directly, but, unfortunately, one has not been identified. To address this question indirectly, NAF was pulse labeled for 15 min, and the intracellular oligomeric forms of NAF were examined after varying chase times on sucrose gradients. Fractions were immunoprecipitated with the polyclonal Tokyo NA antibody, and the polypeptides were analyzed under nonreducing conditions on SDS-PAGE. As shown in Fig. 8, NAF existed mostly as a monomer with a small amount of disulfide-linked dimer after the 15 min pulse label. After 30 min of chase period more disulfide-linked dimer had formed, and a very small amount of a NAF tetramer species (fractions 5 and 6) consisting of two pairs of disulfide-linked dimers had formed. After 60 min of chase period, the relative ratio of NAF monomers, dimers, and tetramers had not changed dramatically although the absolute amount of the species had decreased because NAF is secreted by this time (see Fig. 4). In addition, the relative amount of each intracellular species is very similar to the relative amounts of the extracellular NAF species. Thus, no evidence for the formation of any intracellular nondisulfide-linked dimers or tetramers was obtained. The interpretation of all these data that we favor is that some of the NAF molecules are secreted in a monomeric and immaturely folded form.

### *Discussion*

The class II integral membrane protein influenza virus NA was modified such that its signal/anchor domain was replaced with the hydrophobic FRED domain of the paramyxovirus F protein. The data indicate that the FRED is capable of acting as a signal sequence and targets the hybrid molecule, NAF, to the lumen of the ER. This result was not unanticipated since it has been reported that a great many hydrophobic sequences can replace the signal sequence of yeast invertase (20). Previous work has shown that signal/anchor



**Figure 8.** Kinetic analysis of the oligomeric forms of intracellular NAF on sucrose density gradients. NAF recombinant virus-infected CV1 cells were labeled with Tran[<sup>35</sup>S] label for 15 min and incubated in chase medium. At varying times, the intracellular NAF species or, after 3 h, the secreted NAF species (SEC) were fractionated on sucrose density gradients as described in Materials and Methods. Fractions were collected from the bottom of the gradients and immunoprecipitated with polyclonal Tokyo NA serum, and the polypeptides were analyzed on SDS-PAGE under nonreducing conditions. D and M mark the approximate position of the various disulfide-linked dimeric and monomeric species, respectively.

domains of class II integral membrane proteins are sufficient to target and anchor heterologous proteins in the ER membrane (18, 59) and that some foreign transmembrane domains can take the place of a natural signal/anchor domain

(58). Thus, since the FRED domain has been shown to function as a membrane anchor when transposed to the COOH terminus of a soluble molecule (37), it was of interest to determine whether it had the ability to anchor NAF stably in

membranes. Proteolytic cleavage of the FRED at a site predicted to be an excellent site for recognition by signal peptidase (52) precluded the determination of whether FRED could act as a combined signal/anchor. The FRED domain may have failed to act as an anchor because it was not sufficiently hydrophobic and was pulled across the bilayer with cleavage occurring secondarily. Alternatively, the FRED may not have anchored because the cleavage site was exposed to a proteolytic activity (presumably signal peptidase) in the lumen of the ER during translocation of the nascent NAF chain. A cryptic site for cleavage in a signal/anchor domain was identified in invariant chain (I $\gamma$ ) when the cytoplasmic tail was deleted, which may have affected its membrane anchoring ability (29). In the natural F protein, where the FRED is in an internal position in the molecule, it is translocated across the ER membrane and remains uncleaved. However, an explanation for the different cleavage susceptibilities could be that if the NAF protein FRED is inserted into the ER membrane as a loop (3, 48, 55) then it would be inverted in the membrane as compared with its orientation in the native F protein. It has been proposed for some hydrophobic domains that the difference between their ability to act as a signal sequence or a signal/anchor domain is due to the presence or absence of a site for cleavage by signal peptidase (48). We have chosen not to alter the cleavage site within FRED because then it would no longer be the natural domain.

The data indicate that NAF is a soluble molecule in the ER that is efficiently secreted from the cell ( $t_{1/2} \sim 90$  min), and this rate of secretion is very similar to that found for transport of NA to the cell surface (5; Paterson, R. G., and R. A. Lamb, unpublished observations). It is widely believed that the rate-limiting step in transport in the exocytotic pathway is the variable exit times of soluble and membrane-bound proteins from the ER, whereas the rate of transport for all proteins from the Golgi apparatus to the cell surface is similar (12, 31, reviewed in 30, 43). In the exocytotic pathway, protein disulfide bond formation is catalyzed by protein disulfide isomerase, a resident protein of the ER (see 6, 13). The observed low but steady-state level of NAF disulfide-linked dimers for 60 min after a short pulse label, coupled with an increasing accumulation of dimeric NAF in the medium, is compatible with a rapid rate of intracellular transport after dimer formation. Similarly, the inability to detect all but small amounts of intracellular NAF species that had acquired resistance to Endo H digestions whereas the majority of extracellular NAF species were resistant to Endo H digestion, supports the idea of rapid transport from the medial-Golgi apparatus to the cell surface.

In other studies on the maturation of integral membrane proteins, mAbs that recognize protein conformation have proven most useful to study the kinetics of folding and oligomerization of integral membrane proteins, especially HA of influenza virus, the G glycoprotein of vesicular stomatitis virus (VSV G) and the hemagglutinin-neuraminidase (HN) of the paramyxovirus SV5 (7, 8, 33, 56). Folding of the polypeptides towards a native structure is thought in the large part to occur in the ER together with the oligomerization process. With NAF, very little reactivity of the intracellular molecules could be obtained with conformation-specific mAbs, whereas a greater reactivity could be gained with the extracellular molecules. Again, these findings are compatible

with a rapid rate of transport after folding of the molecules to a form recognizable by the mAbs. More surprising was the finding that the majority of extracellular NAF molecules had not assumed a form that was recognizable by the mAbs.

When the oligomeric form of the extracellular NAF species was examined, it was found that there was a mixture of different types of species. The majority of molecules were in the form of monomers and dimers; however, only a small population of these molecules could be recognized by conformation-specific mAbs. An even smaller amount of NAF was found to have matured to a tetrameric form that had both enzymatic activity and was recognized by an mAb that inhibits enzyme activity. Although native folding and oligomerization may be a prerequisite for transport of many proteins out of the ER (5, 7, 8, 16, 23), the data shown here are compatible with the view that incompletely folded and nontetrameric forms of NAF are transported intracellularly and are secreted from the cells. However, we cannot completely exclude the remote possibility that oligomeric NAF dissociates and denatures on release into the medium. We have not investigated the possibility that the different NAF forms have varying transport rates, and, thus, the  $t_{1/2}$  of 90 min for secretion reported here represents an average for all the NAF species. However, virtually all NAF molecules are secreted in 4 h, and there was no evidence of extensive turnover of species in the ER. Other examples of misfolded or not fully folded proteins that are transported to the cell surface include a chimeric molecule consisting of the HA ectodomain and the VSV G transmembrane domain and cytoplasmic tail (44) and the various forms of a membrane-anchored form of pyruvate kinase (18).

Several models for the mechanism by which proteins could be sorted in the exocytotic pathway have been proposed (reviewed in 41). For some time it was thought that transport of proteins to the cell surface would be mediated through receptors that recognize positively acting signals. Latterly, it has been suggested, with some evidence provided, that transport of molecules to the cell surface is by default (bulk flow) and that sorting is achieved by selective retention of proteins in the ER or Golgi apparatus (35, 41, 45). Recently, it has been shown that the paramyxovirus HN glycoprotein specifically and transiently interacts with the resident ER protein GRP78-BiP. The duration of this interaction correlates with the rate of HN folding and oligomerization (36), suggesting that this is a normal role of GRP78-BiP as was previously proposed for its interaction with immunoglobulin heavy chains (4). In addition, many mutant proteins (both soluble and membrane bound) that do not fold and oligomerize properly, form a long-lived interaction with GRP78-BiP that presumably prevents their further intracellular transport (9, 16, 21, 34). We suggest that the NAF molecules that are transported in an incompletely folded and nontetrameric form escape retention in the ER because the hypothetical retention signals in NAF are buried at an early stage in the complex folding process. A possible interaction of NAF with GRP78-BiP remains to be investigated.

For those integral membrane proteins where it has been shown that folding and oligomerization are prerequisites for intracellular transport, it has not been possible to uncouple all the stages of folding from oligomerization. The formation of these oligomers may in part involve their membrane anchorage domains and their degree of fluidity in the lipid

bilayer. It would also be expected that the rate of oligomerization is dependent on the relative concentration of the individual polypeptide chains. With NAF, we do not know when the FRED domain is cleaved with respect to completion of synthesis of the nascent chain or folding of the molecule. However, cleavage before the oligomerization process begins could well perturb normal folding and make oligomerization kinetically unfavorable since the molecules are no longer fixed in two dimensions but are in solution. Thus, these data suggest that there may be significant differences in the requirement for oligomerization between membrane-bound and secretory glycoproteins for their intracellular transport; it is known that several naturally secreted proteins are monomeric (see 9 and references within). In two previous reports describing soluble forms of formerly membrane-bound proteins, extracellular monomers have been found, but the interpretation of these data is complicated by several factors. In studies with soluble forms of influenza virus HA (7, 15, 16, 50), differing results have been obtained. In one case, secreted HA was shown by cross-linking studies to be trimeric (16), whereas, in the other case, HA was found to be secreted in a monomeric form (7). These differences could be due to the fact that different HA subtypes were used, reflecting subtle differences in the HA molecules, or, alternatively, because the HA oligomer dissociated on sucrose gradients. In addition, although a monomeric anchor-minus and secreted form of the normally trimeric Rous sarcoma virus envelope glycoprotein has been observed on sucrose density gradients, the monomers are thought to have arisen by dissociation of trimers, probably due to the centrifugation conditions used (10). Lastly, it is not known if formation of the intermolecular disulfide bond in the stalk region of the NAF molecule is a prerequisite or consequence of oligomerization. In the influenza virus NA, the degree of interaction between the four subunits is extensive and involves both hydrophobic and salt-link interactions. The outside strands of two  $\beta$  sheets on neighboring subunits are sufficiently close together that their interaction has been characterized as a continuation of the  $\beta$  sheet across the interface (51). Thus, our data on NAF emphasizes that oligomerization is a complex process that depends on several factors and once it has occurred it is unlikely to be easily reversible.

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