Addition of Carbohydrate Side Chains at Novel Sites on Influenza Virus Hemagglutinin Can Modulate the Folding, Transport, and Activity of the Molecule

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Abstract. We have constructed and expressed a series of mutant influenza virus hemagglutinins, each containing a new consensus site for glycosylation in addition to the seven sites found on the wild-type protein. Oligosaccharide side chains were added with high efficiency at four of the five novel sites, located on areas of the protein's surface that are not normally shielded by carbohydrate. Investigations of the structure, intracellular transport, and biological activities of the mutant hemagglutinin molecules indicated that (*a*) supernumerary carbohydrate side chains can be used to shield or disrupt functional epitopes on the surface of hemagglutinin, and (*b*) the presence of an additional oligosaccharide may cause temperature-dependent defects in the transport of the glycoprotein. We discuss the addition of supernumerary oligosaccharides as a general tool for shielding chosen areas of the surface of proteins that enter or traverse the secretory pathway.

THE functions of the carbohydrate side chains that are covalently attached to the majority of eukaryotic membrane and secretory proteins are not yet fully understood. It has been suggested that N-linked oligosaccharides may play a variety of roles including (*a*) the maintenance of the structure and stability of glycoproteins, (*b*) the direction of proteins to various subcellular compartments both after biosynthesis and for degradation, and (*c*) recognition functions in specific adhesion of cells and tissue organization during development or neoplasia (for reviews see Gibson et al., 1980; Elbein, 1981, 1987; Olden et al., 1985). Studies using drugs that are specific inhibitors of the addition or modification of N-linked oligosaccharide side chains have been performed in attempts to define the functional roles of these carbohydrate moieties (for review see Elbein, 1987). The advent of recombinant DNA technology has facilitated studies involving the removal of individual glycosylation sites via oligonucleotide mutagenesis (Machamer et al., 1985; Miyazaki et al., 1986; Santos-Aguado et al., 1987; Guan et al., 1988; Matzuk and Boime, 1988). The majority of these investigations have indicated that oligosaccharide side chains indeed play a role in glycoprotein structure and stability, with different proteins being more or less dependent on the presence of their carbohydrate moieties (Elbein, 1987). Oligonucleotide-directed mutagenesis has also been used to cause the addition of oligosaccharide chains at novel positions on nascent polypeptides, which in some but not all cases promoted the intracellular transport of mutant proteins that were previously blocked in transport along the secretory pathway (Guan et al., 1985; Machamer et al., 1985; Machamer and Rose, 1988*a*, *b*). Because the three-dimensional structures of these proteins were not known, it was not possible to predict whether the novel side chains would be attached at positions normally located on the surface or within the interior of the wild-type protein. In this paper we have taken advantage of the knowledge of the precise three-dimensional structure of the hemagglutinin (HA)[1] of influenza virus to introduce novel oligosaccharide consensus sequences at chosen sites on the surface of the protein.

HAs comprise a large family of highly polymorphic glycoproteins which may vary up to 60% in amino acid sequence while still retaining very similar structural and biological properties (Wilson et al., 1981; Wiley et al., 1981). This extensive sequence variation, which allows newly evolved viruses to escape immunity acquired by the host during previous infection, may either occur gradually by the accumulation of single amino acid changes (antigenic drift), or abruptly after the introduction of a novel HA gene from an animal reservoir (antigenic shift) (reviewed by Air et al., 1987). There is also considerable variation in the position and number (four to nine) of potential carbohydrate attachment sites on the different HA molecules (Klenk, 1980; Wilson et al., 1983; Keil et al., 1984; Wilson, I. A., unpublished observations). Occasionally, when antigenic shift or drift creates a novel consensus sequence (Asn-X-Ser or Thr) for N-linked glycosylation, the addition of the new oligosac-

1. *Abbreviations used in this paper*: endo H, endoglycosidase H; ER, endoplasmic reticulum; HA, hemagglutinin; HA0, core-glycosylated precursor form; XHA, wild-type X31 hemagglutinin.

charide may modulate the immune recognition of the molecule by shielding an antigenic epitope (Wiley et al., 1981; Caton et al., 1982; Raymond et al., 1986). This strategy for evasion of immune recognition has been reproduced in the laboratory; a virus selected by growth in the presence of an anti-HA mAb was found to bear an altered HA containing an additional oligosaccharide side chain (Skehel et al., 1984). This side chain was attached near to, but not within, the target antigenic epitope. Because evasion of the immune system appears to be the major evolutionary pressure on influenza virus, it might be expected that, with time, oligosaccharide side chains would progressively cover more of the surface of the HA molecule. Comparison of the carbohydrate attachment sites on a large number of HA variants reveals that, although the majority of the protein's surface has been shielded by an oligosaccharide at one time or another during the evolution of the molecule, five surface areas appear always to have lacked carbohydrate.

Why has there been selection against the presence of oligosaccharide side chains within these five carbohydrate-free surface regions? In only one case is there an obvious reason for the absence of the N-linked side chain. At the apex of the molecule, near the contact points between the globular domains of the three subunits, there is a sialic acid–binding site involved in the attachment of virus to host cells (Wilson et al., 1981). Carbohydrate residues shielding this region would mask the binding site and abolish infectivity. We postulate that other regions of HA that must remain devoid of carbohydrate might include recognition site(s) for putative cellular proteins involved in the initial folding and mobilization of HA into the secretory pathway (e.g., BiP; Gething et al., 1986*b*), and/or in the directional transport of HA to the apical surface of polarized epithelial cells (Rodriguez-Boulan and Sabatini, 1978; Blobel, 1980; Fitting and Kabat, 1982). Alternatively, exclusion of carbohydrate from surface areas might occur for structural reasons. Attachment of a carbohydrate side chain at some positions could interfere either with correct folding and oligomerization of the molecule (Gething et al. 1986*b*), or with assembly of HA into infectious virions.

To analyze these and other possibilities we have used site-directed mutagenesis to introduce nucleotide sequences encoding novel glycoslyation sites into the cloned copy of the gene encoding the X31 HA. This paper described the characterization of five mutant glycoproteins each of which has a novel consensus sequence located within one of the carbohydrate-free regions on the surface of the molecule. We have shown that the addition of a supernumerary carbohydrate side chain can be used to shield or disrupt functional epitopes on the surface of the HA molecule. We have also found that the presence of a novel side chain can cause temperature-dependent defects in the intracellular transport of the glycoprotein. However, these transport defects result from generalized effects on the folding of the HA molecule, rather than from shielding of a specific transport epitope on the surface of the protein.

Materials and Methods

Selection of Novel Consensus Sequence Sites

The three-dimensional structure of the A/Aichi/68 (X31) HA (Wilson et al., 1981) was displayed on an Evans and Sutherland color multi-picture system. Analysis of the protein structure was performed using a Digital Electronic Corp. (Marlboro, MA) VAX 11/750. The structure representations were projected using GRAMPS (O'Donnell and Olson, 1981) and GRANNY (Connolly and Olson, 1985) programs. The HA1 amino acid sequences reported for 47 HA variants and the HA2 sequences reported for 22 of these variants were collated and the positions of all the potential glycosylation sites on the HA proteins were noted. The positions of all the residues that have, in one sequence or another, been the asparagine of a canonical glycosylation sequence were amassed on the X31 structure and the projected surface areas that would be masked by the attached side chains were highlighted. Areas of the surface of the HA trimer which appeared never to have been shielded by carbohydrate were noted and approximate locations were selected for the attachment of novel oligosaccharides that would cover these areas. Precise selection of residues to be altered to yield new canonical sequences using the criteria described in the results section was made using a three-dimensional molecular model based on the coordinates determined by x-ray crystallography (Wilson et al., 1981) and constructed by John Mack.

Recombinant DNA Techniques

Buffers and reaction conditions for restriction enzymes, T4 DNA ligase, and the Klenow fragment of DNA polymerase I were those listed by the commercial source New England Biolabs (Beverly, MA). Isolation of DNA fragments, preparation of plasmid DNAs, and other standard recombinant DNA techniques were carried out as described (Sambrook et al., 1988, and in other references cited therein). Transformation of *Escherichia coli* DH1 or TG1 cells was done by the method of Hanahan (1983).

Site-specific Mutagenesis of HA cDNA Using Mismatched Oligonucleotides

Plasmid pSVEXHA (which contains a full length cDNA copy of the HA gene from influenza virus strain A/Aichi/68 [Doyle et al., 1986]) was digested with Pst I and Bam HI restriction endonucleases, and a 2,579-bp DNA fragment (including 771 bp of pBR322 sequences that lie between the Pst I and Cla I restriction sites and 1,608 bp encoding the ectodomain of X31 HA) was purified by gel electrophoresis. This fragment was inserted between the Pst I and Bam HI site of the double-stranded replicative form of M13mp19 phage DNA, and *E. coli* TG1 cells were transfected with the ligated DNA molecules. A recombinant phage was selected in which the single-stranded DNA purified from M13 virions secreted from infected bacterial cells contained the coding sequences of HA cDNA. Six 20-mer oligonucleotides were synthesized to be complementary, except for the required mismatches, to HA cDNA sequences encoding regions of the protein where amino acid substitutions were planned to introduce novel Asn, Ser, or Thr residues. The procedures used to carry out oligonucleotide-directed mutagenesis and identification of the desired mutants have been described in detail in Zoller and Smith (1983, 1985) and in Doyle et al. (1986). Once the desired base substitutions had been confirmed, the double-stranded replicative form of the phage DNAs containing each mutant HA sequence was prepared and purified by centrifugation on $CsCl_2$ gradients. The 1,608-bp Cla I–Bam HI DNA fragment encoding the X31 HA ectodomain was isolated and used to replace the equivalent wild-type sequences in the SVEXHA recombinant viral genome.

Generation of SV40–HA Recombinant Virus Stocks and Infection of CV-1 Cells

SV40–HA recombinant genomes containing wild-type and mutant forms of the X31 HA gene were transfected into CV-1 cells using DEAE–dextran and chloroquine as previously described (Doyle et al., 1985). High-titer virus stocks were developed and used to infect fresh monolayers of CV-1 cells for analysis of the biosynthesis and intracellular transport of the wild-type and mutant HAs. Conditions for growth and infections of CV-1 cells were as described previously (Doyle et al., 1985).

Analysis of the Biosynthesis and Intracellular Processing of Wild-type and Mutant HAs

Analysis of the biosynthesis of wild-type and mutant HA proteins at 37°C was carried out by pulse-chase radiolabeling of infected cells with [^{35}S]methionine as described by Gething et al. (1986*b*). When it was desired to block N-linked glycosylation of the HA molecules, tunicamycin (5 μg/ml)

was added to the medium for 2 h before labeling and throughout the labeling procedure. Cell extracts were immunoprecipitated with a polyclonal anti-X31 HA rabbit antiserum as described by Gething et al. (1986*b*), or with mAbs as previously described by Webster et al. (1983). The mAbs used in this study (11/4 [A site], 17/2 [B site], 69/1 [C site], and 78/6 [D site]) were generously provided by R. Webster, St. Jude Children's Research Hospital, Memphis, TN. Digestion with endo-*N*-acetylglucosaminidase H (endo H) was carried out as described (Owen et al., 1980). Binding of guinea pig, human, turkey, or chicken erythrocytes to HA expressed on the surface of infected CV-1 cells was performed as described previously (Doyle et al., 1985). The cellular location of wild-type and mutant forms of HA was determined by indirect immunofluorescence of infected cells (Doyle et al., 1985; Ash et al., 1977) using the polyclonal and monoclonal antibodies described above.

Analysis of the biosynthesis of the wild-type and mutant proteins at 30 and 42°C was carried out as described for 37°C, with the following modifications. CV-1 cells were infected with recombinant viruses and incubated at 37°C for 36–40 h. Before labeling, the infected cells were preincubated at either 30°C for 2 h or at 42°C for 30 min. During the labeling and chase periods, these temperatures were accurately maintained until cell extracts were prepared. Cell fusion, erythrocyte binding, and indirect immunofluorescence assays which require an accumulation of sufficient HA on the plasma membrane for determination of biological activity were carried out with the following modifications. CV-1 cells infected with recombinant viruses were incubated at 37°C for 18 h. At this time, just before the commencement of synthesis of HA, the infected cells were incubated at 30°C for an additional 48 h to ensure that all HA appearing and accumulating on the plasma membrane had been synthesized at 30°C.

Enumeration of the Oligosaccharide Side Chains on HA Molecules Using Partial Digestion with Endo-N-acetylglucosaminidase H

Infected cells were pulse labeled with [^{35}S]methionine for 5 min at 37°C. The monolayers were then washed once with Tris-buffered saline and cell extracts were prepared as described previously (Gething and Sambrook, 1981). Proteins immunoprecipitated using anti-HA serum were digested with endo H (ICN Immunobiologicals, Inc., Irvine, CA; 1.0 mIU per sample) as described by Owen et al., 1980 for various times between 0 min and 3 h. At each time point an aliquot was removed and added to an equal volume of gel sample buffer (Gething et al., 1986*b*). The samples were then analyzed by SDS-PAGE and autoradiography.

Protease Sensitivity of Wild-type and Mutant HA Molecules

Cell extracts were prepared from infected cells using a lysis buffer lacking protease inhibitors (50 mM Tris-HCl, pH 8.0, containing 1% NP-40). Aliquots of the extracts were incubated on ice for 15 min either in the absence or presence of 25 μg/ml trypsin before the addition of a twofold excess of soybean trypsin inhibitor. After immunoprecipitation using anti-HA sera, the digestion products were analyzed by SDS-PAGE and autoradiography.

Results

Identification of Surface Regions on HA That Normally Lack Carbohydrate and Choice of Novel Sites for Glycosylation

Areas on the surface of the HA molecule that have never been covered by an oligosaccharide in any known influenza virus variant were identified by locating on a molecular graphics display of the X31 HA structure the positions of all potential glycosylation sites within the sequences of HAs of 47 different influenza A virus strains (see Materials and Methods). These sites can be grouped into three classes: those whose position is conserved in all HA molecules, those whose general location on the protein surface remains relatively constant although the specific point of linkage to the polypeptide chain may vary, and those that are attached at quite different positions on different molecules. The solvent-accessible surface area that would be "masked" by the oligosaccharides was estimated and highlighted on the molecular graphics display. Several surface areas which were never glycosylated were distinguished.

We wished to introduce novel consensus sequences (Asn-X-Ser/Thr) so as to maximize the shielding of the carbohydrate-free regions while minimizing disruption of the structure of the HA trimer. The choice was first limited to amino acids that lay in the center of each barren region and were clearly exposed on the surface of the protein. Secondly, amino acids that were highly conserved between different HAs or that appeared to play an important role in maintaining the structure of the protein were excluded. The final criterion was that the residue designated for attachment of the extra oligosaccharide should have its side chain directed outward from the surface of the protein so as to minimize structural distortion caused by the addition of the oligosaccharide to the ε-amino group of the novel or natural asparagine residue. The positions on the X31 HA molecule that were chosen for attachment of supernumerary carbohydrate side chains and the amino acid changes that were introduced by site-directed mutagenesis to create the novel glycosylation sites are shown in Table I and in the summary Fig. 7. Within two of the barren areas, at residues 54 and 188, a suitable asparagine was available whose side chain was directed outward; the consensus site could be introduced by substituting a threonine or serine residue for the amino acid located two residues further along the polypeptide chain. Because the presence of proline at the central position of the consensus site has been reported to prevent glycosylation (Neuberger et al., 1972), a secondary mutant (54B) was constructed in which proline 55 was substituted by glycine, the amino acid which is present in this position in the majority of HA molecules whose sequence is known. In two other barren areas, threonine or serine residues lay two amino acids downstream of residues (46 and 225) that were suitable for substitution by asparagine. In the final barren region, both the asparagine (at position 476 [HA2 position 147]) and the threonine residue had to be introduced by mutagenesis.

Table I. Nucleotide Substitutions and Corresponding Amino Acid Changes in Mutants with Supernumerary Consensus Sequences for Glycosylation

Name	Sequence of wild-type	Sequence of mutant
XHA46+	Ser-Ser-Thr TCC-TCA-ACG	**Asn**-Ser-Thr **AAC**-TCA-ACG
XHA54A+	Asn-Pro-His AAT-CCT-CAT	Asn-Pro-**Thr** AAT-CCT-**ACT**
XHA54B+	Asn-Pro-Thr* AAT-CCT-ACT	Asn-**Gly**-Thr AAT-**GGT**-ACT
XHA188+	Asn-Gln-Glu AAC-CAA-GAA	Asn-Gln-**Ser** AAC-CAA-**ACA**
XHA225+	Gly-Leu-Ser GGT-CTG-TCT	**Asn**-Leu-Ser **AAT**-CTG-TCT
XHA476+	Ala-Cys-Ile GCT-TGC-ATA	**Asn**-Cys-**Thr** **AAT**-TGC-**ACA**

* DNA from the XHA54A+ mutant was used as the template for mutagenesis to generate the XHA54B+ mutant.

Construction and Expression of Mutant HAs Containing Novel Glycosylation Sites

Oligonucleotide-directed mutagenesis of cloned X31 cDNA was used to introduce the nucleotide changes (Table I) required to encode the novel consensus sites for glycosylation. To analyze the phenotypes of the mutant glycoproteins, SV40–HA recombinant genomes were constructed in which either the wild-type X31 HA gene (XHA) or the mutant genes (46+, 54A+, 54B+, 188+, 225+, and 476+) replaced the coding sequences of the late region of SV-40 DNA. These recombinant genomes were transfected into simian CV-1 cells and high titer recombinant virus stocks were developed. After infection of fresh monolayers of CV-1 cells with these stocks, a number of different assays were performed to analyze the phenotypes of the wild-type and mutant XHA proteins. The details of all these procedures are given in Materials and Methods.

Analysis of the Number of N-linked Side Chains on Wild-type and Mutant XHA Molecules

To determine whether the novel consensus sequences were recognized as additional sites for N-linked glycosylation of the nascent polypeptide chain, the electrophoretic mobility of the core-glycosylated precursor form (HA0) of each of the mutant proteins was compared with that of wild-type XHA. Fig. 1 *A* shows the results obtained when cells expressing the wild-type and mutant proteins were labeled with [^{35}S]methionine at 37°C. The major protein band precipitated from the labeled cell extracts with anti–HA serum corresponds to HA0 (80 kD). A fainter band (~55 kD) corresponds to the core-glycosylated HA1 subunit, generated by partial proteolysis of newly synthesized monomeric HA0 after preparation of cell extracts (our unpublished data). The autoradiograph also reveals a protein species (~77 kD) that coprecipitates with the HA polypeptides. We have previously identified this protein, which is seen to associate to greater or lesser extents with the wild-type and mutant forms of HA in all the autoradiographs presented in this paper, as the immunoglobulin heavy chain binding protein BiP, a cellular protein resident in the lumen of the endoplasmic reticulum (ER) (Bole et al., 1986). BiP interacts with HA molecules until they become assembled into their native trimeric structure and are transported via the Golgi apparatus to the cell surface (Gething et al., 1986*b*).

The HA0 forms of mutants 46+, 54B+, 188+, and 476+ labeled during a 5-min pulse migrated more slowly than wild-type HA0 (Fig. 1 *A*). However, the wild-type and mutant proteins synthesized in the presence of tunicamycin (Fig. 1 *B*) migrated with identical apparent molecular masses, indicating that the differences in mobility of the HA0 species seen in Fig. 1 *A* reflect changes in the complement of carbohydrate side chains rather than alterations to the polypeptide backbone. The presence of additional carbohydrate side chains on these mutants is more apparent after cleavage of the precursor into HA1 and HA2 subunits (see below; Fig. 3). As expected, increased mobility is displayed by the HA1 subunits of mutants 46+, 54B+, and 188+, and by the HA2 subunit of mutant 476+.

The glycosylated forms of mutants 54A+ and 225+ migrated with the same mobility as the wild-type HA (Fig. 1 *A*) suggesting that the new consensus sequences were not used for glycosylation. It is likely that a turn in the polypeptide chain caused by the proline residue in the Asn_{54}-Pro-Thr sequence prevents simultaneous recognition of the asparagine and threonine residues by the glycosyl transferase (Bause, 1983), since substitution of glycine for proline resulted in supernumerary glycosylation of the 54B+ mutant. The structural basis for the lack of use of the novel site in the 225+ mutant is less easily understood. Inspection using molecular graphics of the positions of amino acids in the vicinity of residue 225 in the final conformation of the wild-type protein reveals that the aromatic ring of Trp 222 lies 4.9 Å above the C_α atom of Gly 225. Since we do not know the conformation of the mutant protein at the time of glycosylation, it is difficult to judge whether this bulky residue could sterically hinder the access of the glycosyl transferase to the introduced asparagine residue in mutant 225+.

Accurate analysis of the number of carbohydrate side chains on each mutant was carried out as described by Schuy et al. (1986) by partial digestion with endo H of HA molecules pulse-labeled for 5 min at 37°C, followed by separation of the reaction products by SDS-PAGE. Examples of these analyses for the wild-type and two of the mutant HAs (46+ and 476+) are presented in Fig. 2. At each time point a series of discrete bands are seen, each differing by one N-linked oligosaccharide, and the total number of side chains on the wild-type or mutant HA molecules can be determined by counting the total number of bands. We confirmed the previous reports of seven N-linked side chains on the XHA (Nakamura and Compans, 1979; Wilson et al., 1981). Mutants 46+, 54B+, 188+, and 476+ each contained eight side chains, verifying that the novel consensus sequence in each of these proteins was used efficiently. Mutants 54A+ and 225+ contained only seven N-linked side chains.

Analysis of the Intracellular Transport and Processing of the Wild-type and Mutant XHA Proteins

In a standard pulse-chase protocol, acquisition by a glycoprotein of resistance to cleavage of its N-linked oligosaccharides by endo H provides a convenient measure of transit time from the ER into the medial Golgi cisternae (Kornfeld and Kornfeld, 1985). The mutant proteins displayed extensive variation in their rate of acquisition of resistance to the glycosidase after synthesis at 37°C. Densitometric analysis of the autoradiographs from these experiments allowed quantitation of the percentage of HA molecules that had become resistant to endo H at the different times after synthesis (Table II). Two mutants (46+ and 225+) were processed at rates very similar to that of the wild-type protein. Mutants 54A+, 54B+, and 188+ were processed more slowly and mutant 476+ showed very little conversion (~7% during a 2-h chase period) to a form resistant to the glycosidase, suggesting that this mutant protein is largely restricted to the ER.

The time course of appearance of newly synthesized protein on the plasma membrane was assayed by analyzing its accessibility to proteolysis by trypsin added to the external medium (Doyle et al., 1986). Previous experiments have shown that >90% of the radiolabeled, wild-type XHA protein becomes accessible to exogenously added trypsin within 2 h of chase (Doyle et al., 1986). The results of the analysis of the wild-type and mutant glycoproteins after a 2-h period of chase at 37°C are shown in Fig. 3 *A*. Densitometric analy-

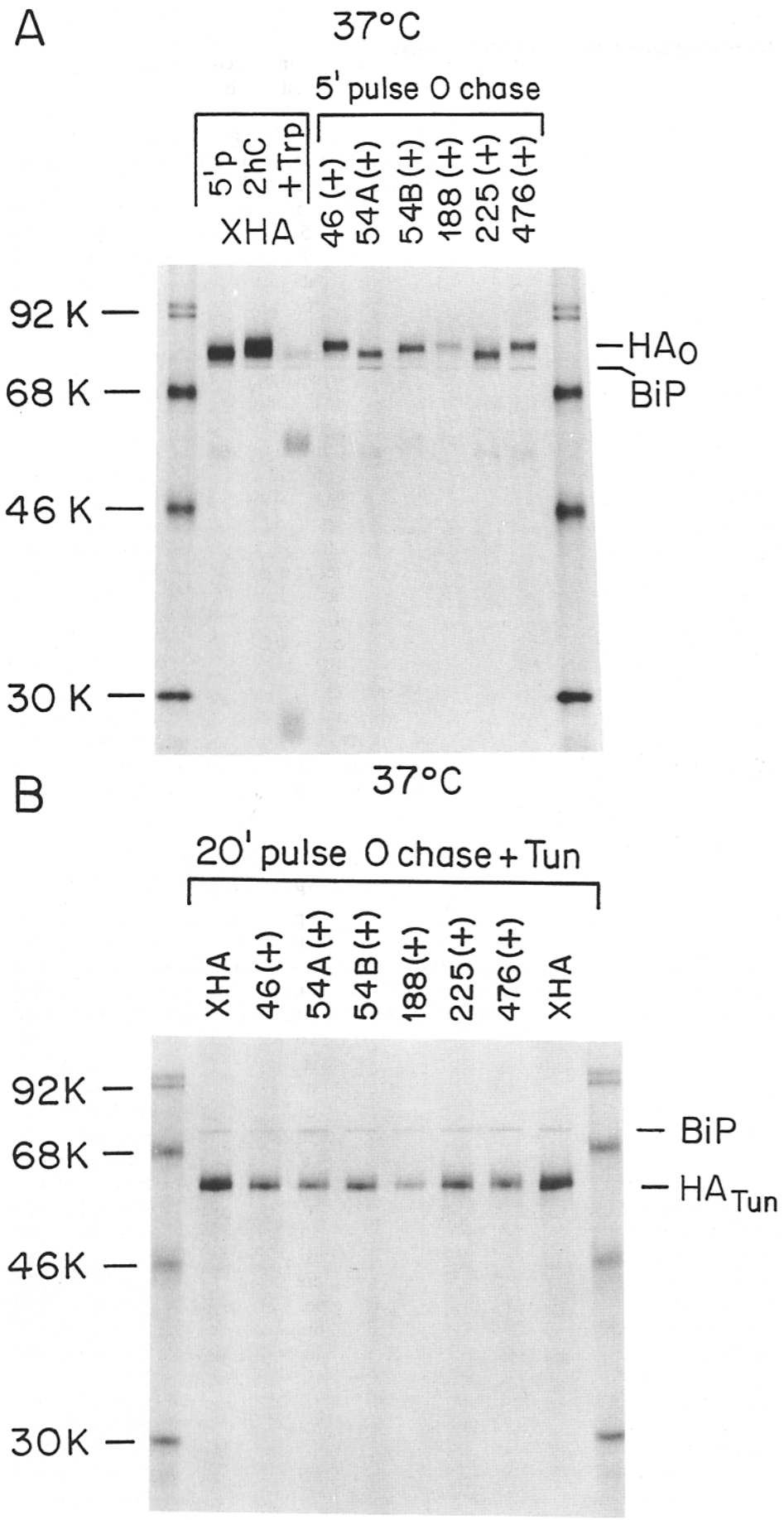

Figure 1. Size comparison of wild-type XHA and mutant proteins containing supernumerary sites for N-linked glycosylation. CV-1 cells infected for 40 h at 37°C with SV40–HA recombinant viruses were labeled for 5 min with [^{35}S]methionine in the absence (*A*) or presence (*B*) of tunicamycin as described in Materials and Methods. Cell extracts were then prepared and HA polypeptides were precipitated with a polyclonal anti–HA serum, separated by SDS-PAGE, and autoradiographed.

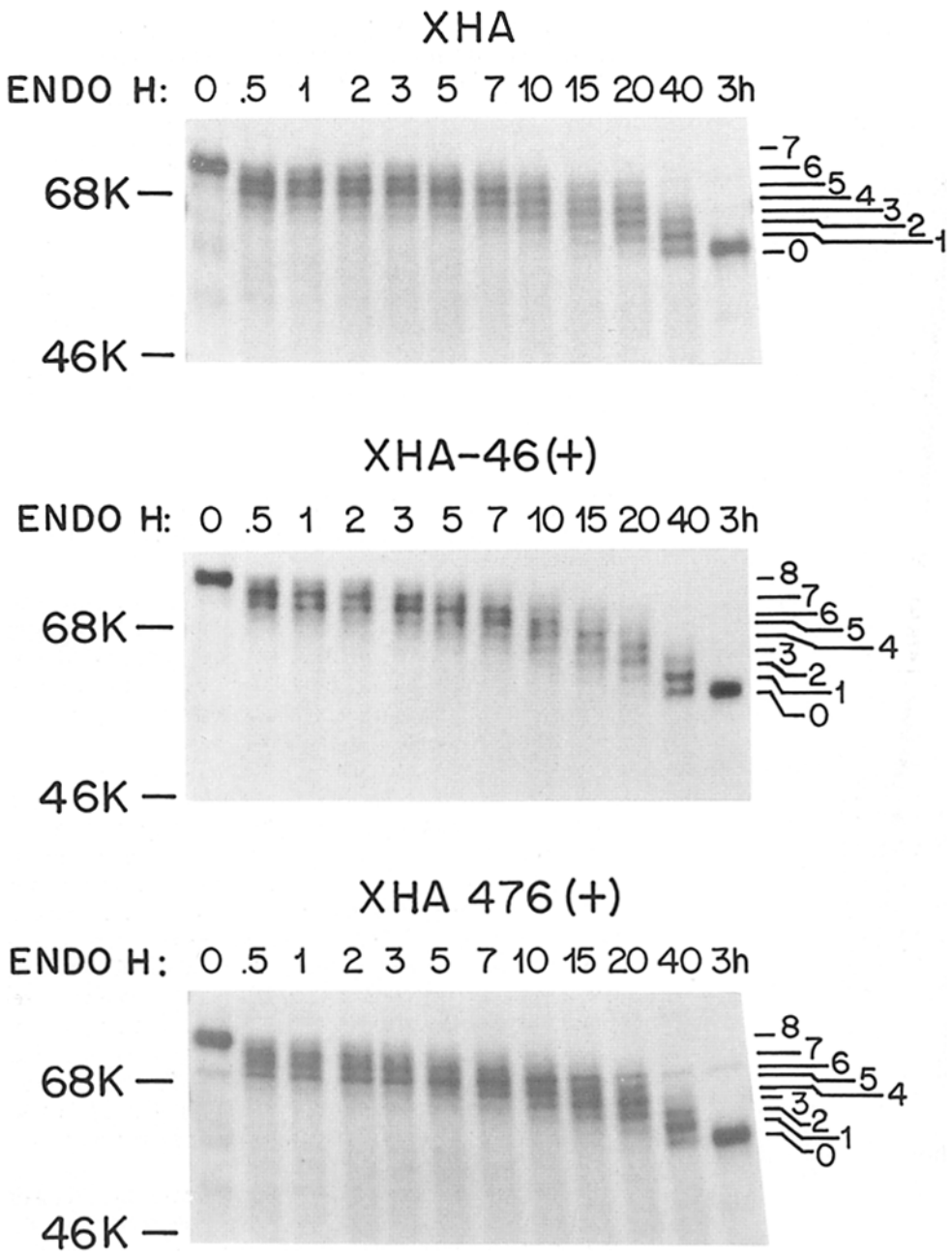

Figure 2. Analysis of the number of carbohydrate side chains on wild-type and mutant XHA proteins by partial digestion with endo H. CV-1 cells infected for 40 h at 37°C with SV40–HA recombinant viruses were labeled for 5 min with [^{35}S]methionine. Cell extracts were then prepared and HA polypeptides were precipitated with a polyclonal anti-HA serum. The immunoprecipitates were then treated for 0.5 min to 3 h with endo H as described in Materials and Methods. The reaction products were separated by SDS-PAGE and autoradiographed. The duration of treatment with endo H is shown in minutes except for the 3-h time point.

sis of the HA0, HA1, and HA2 bands revealed extensive differences in the rate at which the mutants reached the cell surface at 37°C. Mutant 46+ became accessible to trypsin at a rate similar to that of the wild-type protein, while a larger proportion of the other mutants remained inaccessible to the protease at the end of the chase period. Mutant 476+ did not become accessible to trypsin after synthesis at 37°C, a result consistent with its inefficient transport from the ER at that temperature.

The data shown in Fig. 3 *A* also suggest that there may be variation in the stability of the mutant proteins at 37°C since there appears to be some loss of the labeled 54A+ and 188+ bands during the 2-h chase period, presumably due to degradation of these mutant proteins. Extensive turnover of these two mutants and of mutant 54B+ was observed during extended chase periods of up to 24 h at 37°C (results not shown). In addition, the results shown in Fig. 3 indicate that the mature HA0, HA1, and HA2 forms of mutant 188+ migrate more slowly than the equivalent forms of other mutants which contain a supernumerary oligosaccharide. HA 188+ thus appears to undergo more extensive terminal gycosylation than the wild-type protein, due in part to increased modification of some or all of the oligosaccharide moieties and in part to conversion to the complex form of one of the two side chains that normally remain in the mannose-rich form (Doyle et al., 1986; our unpublished results).

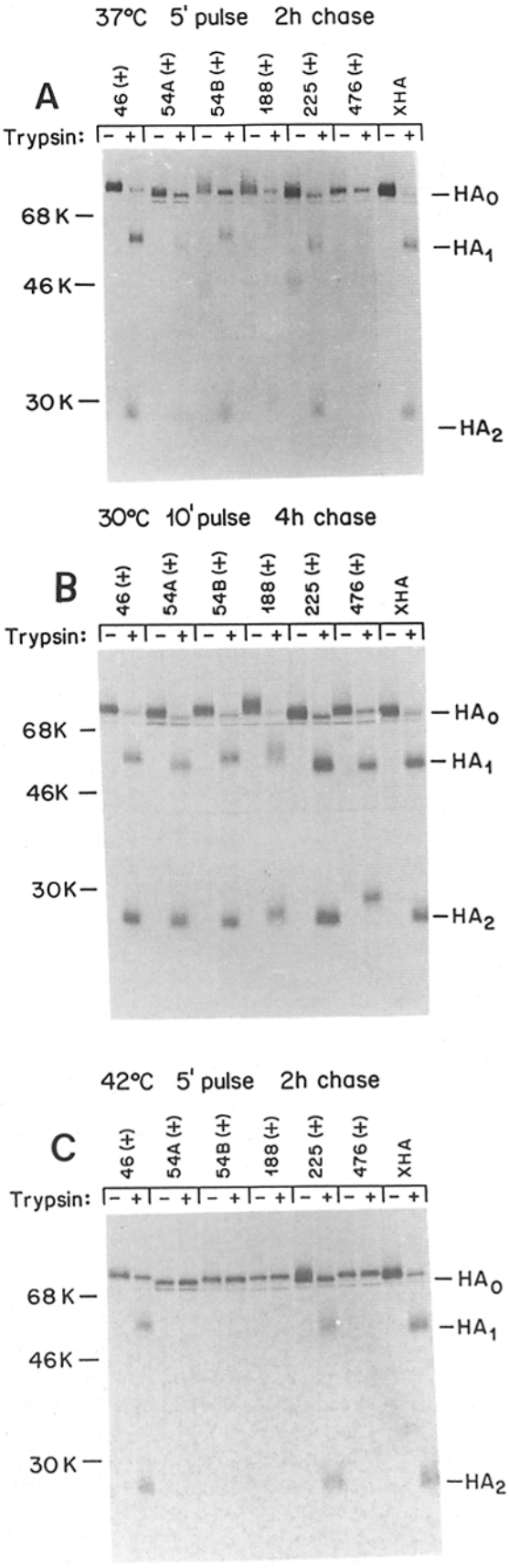

Figure 3. Effect of temperature on the intracellular transport of wild-type and mutant XHA proteins. CV-1 cells were infected for 40 h at 37°C with SV40–HA recombinant viruses. Monolayers of infected cells were pulse labeled with [^{35}S]methionine and then incubated in an excess of nonradioactive methionine, as described in Materials and Methods, either at 37 (*A*), 30 (*B*), or 42°C (*C*). The intact monolayers were then treated with DME (−) or DME containing 5 μg/ml trypsin (+) for 15 min at the appropriate temperature before cell extracts were prepared and analyzed by immunoprecipitation and SDS-PAGE as described in Materials and Methods.

Table II. Results of the Quantitation by Densitometry of the Time Course of Acquisition by Wild-type and Mutant XHA Proteins of Resistance to Endo H

	% HA molecules resistant to endo H		
	15 min p	*+15 min chase*	*+2 h chase*
XHA	0	25	80
XHA46+	0	23	83
XHA54A+	0	16	54
XHA54B+	0	14	59
XHA188+	0	12	77
XHA255+	0	30	76
XHA476+	0	3	7

The accuracy of the densitometry measurements was ±3% in repeated analyses.

Effect of Temperature on the Addition of Supernumerary Oligosaccharide Side Chains and on the Intracellular Transport of the Mutant Proteins

It has been observed that the extent of glycosylation at individual consensus sites can be affected by the temperature at which the protein is synthesized (Schuy et al., 1986), possibly due to alteration of the rate at which the nascent polypeptide folds into a conformation that masks the consensus sequence. We therefore analyzed the wild-type and mutant HA0 proteins after synthesis either at 30°C or at 42°C (Fig. 3, *B* and *C*). Examination of the mobilities of the HA species by SDS-PAGE and autoradiography revealed that there was very little effect of temperature on the use of the natural or supernumerary glycosylation sites on the wild-type or mutant proteins (results not shown). The wild-type HA and mutants 46+, 54+, and 54B+ migrated as single components under all circumstances. Although there appeared to be some minor variability in the efficiency of use of the consensus sequences in the other mutants (188+, 225+, and 476+) at the different temperatures, as evidenced by the appearance of an additional minor component having a mobility corresponding to the addition of one extra or one less oligosaccharide, this minor component did not comprise more than a few percent of the HA molecules.

By contrast, alterations in the temperature had dramatic effects on the transport of the mutant proteins through the cell. When the wild-type and mutant proteins were synthesized at 30°C, the majority of all the labeled proteins became accessible to trypsin within 4 h of chase (Fig. 3 *B*). Most notably, 75% of the molecules of mutant 476+ moved to the plasma membrane where they were cleaved into HA1 and HA2 subunits. On the other hand, after synthesis at 42°C only wild-type XHA and two of the mutants (46+ and 225+) became accessible to trypsin after 2 h of chase (Fig. 3 *C*). The other mutants showed no evidence of processing to the

higher molecular mass, terminally glycosylated form of HA0 indicating that they were not transported from the ER at the higher temperature.

None of the mutants displayed increased turnover after synthesis and transport to the cell surface at 30°C (Fig. 3 *B*). Nor did any of the proteins show evidence of degradation after synthesis at 42°C (Fig. 3 *C*). It is likely that mutants 54A+, 54B+, and 188+ remained intact at 42°C, despite being turned over more rapidly at 37°C, because they were retained in the ER and were not exposed to extracellular proteases. Retention in the ER also explains the lack of proteolysis of mutant 476+ at 37°C, since this mutant is sensitive to degradation by trypsin after preparation of a cell extract (see below, Fig. 6).

Cellular Location of the Wild-type and Mutant Glycoproteins

Indirect immunofluorescence using an antibody that recognizes all forms of HA was also used to analyze the cellular location of the wild-type and mutant proteins after synthesis at 37 or 30°C. At 37°C, permeabilized cells expressing either wild-type XHA or mutants 46+, 54B+, 188+, and 225+ displayed qualitatively similar patterns of labeling (Fig. 4). In addition to diffuse labeling of the cell surface, there is staining of the perinuclear Golgi region as well as reticular staining of the ER; a pattern that is typical of glycoproteins synthesized in the ER and transported to the cell surface via the Golgi apparatus. The intensity of surface labeling appeared to be significantly decreased for the 54B+ and 188+ mutants (Fig. 4), a result consistent with increased extracellular degradation of these proteins at 37°C (see above, Fig. 3 *A*). Staining of nonpermeabilized cells confirmed that reduced amounts of the 54B+ and 188+ mutants were displayed on the plasma membrane (data not shown). Cells expressing mutant 476+ showed no surface fluorescence but displayed a strong reticular staining pattern (Fig. 4). In addition, there appeared to be a concentration of fluorescence in the juxtanuclear region which might be taken as evidence that 476+ molecules were also located within the Golgi cisternae. In this context, the results shown in Table III (above) indicated that ~7% of mutant 476+ became resistant to endo H during a 2-h chase period. However, we have reported previously (Doyle et al., 1986) that similar patterns of immunofluorescence were seen in control experiments in which infected CV-1 cells were stained with an antiserum that reacts specifically with antigens localized only in the ER. Furthermore, Poruchynsky et al. (1985) have shown by electron microscopy that in COS-7 cells, which are derived from the CV-1 cells used in our studies, the Golgi stacks in the juxtanuclear region are spatially intertwined with an extensive ER, so that in this region bright but not totally coincident immunofluorescence can be obtained with probes for either ER or Golgi elements. Taken together, our data is consistent with the majority of the mutant 476+ protein synthesized at 37°C being retained in the ER and inefficiently transported along the secretory pathway. Staining of nonpermeabilized cells confirmed that little or no mutant 476+ was transported to the cell surface at 37°C (data not shown).

After synthesis at 30°C, there was little difference between the patterns of immunofluorescence of the wild-type and mutant proteins, all of which were displayed efficiently on the cell surface (data not shown). This result confirms the conclusion drawn from the pulse-chase analysis (Fig. 3 *B*) that mutant 476+ can be efficiently transported to the plasma membrane at 30°C.

Detection of Structural Alterations Resulting from the Addition of Supernumerary Oligosaccharide Side Chains

The introduction of amino acid changes that ultimately direct the addition of posttranslational modifications may well alter the native structure of the protein. Structural alterations may arise as a consequence of the substitution of the individual amino acid residues, or the addition of the bulky N-linked glycan, or of a combination of these changes. Although we had attempted to minimize structural effects by careful selection of the novel sites for oligosaccharide addition, we wished to assess if there had been any change in the local or global conformation of the mutant proteins.

mAbs prepared against wild-type XHA were used as probes to detect any alterations in four major antigenic epitopes on the mutant proteins. Three of the antigenic epitopes (sites A, B, and C) are located on the globular head of the molecule (see Fig. 7) and in general consist of exposed loops on the surface of the protein (Wiley et al., 1981). Antibodies specific for these sites have been observed to be relatively indifferent to changes in the global conformation of the HA molecule and to recognize both the monomeric and trimeric forms of the protein. However, the fourth epitope (site D) maps in the trimer interface (Wiley et al., 1981) and appears to be displayed only when the molecule is in the fully folded trimeric form. Single amino acid changes (including those that result in addition or loss of an N-linked glycan) can alter or block recognition by mAbs specific for these sites (reviewed by Wiley and Skehel, 1987). Because some of the antigenic epitopes may have been "masked" by the addition of novel side chains within or near the epitope, binding of the antibodies to the mutant proteins was analyzed after synthesis of the protein both in the absence and presence of tunicamycin, a specific inhibitor of N-linked glycosylation (Schwarz and Datema, 1980). Binding of the mAbs to the glycosylated and nonglycosylated proteins was determined both by immunoprecipitation of labeled HA (Fig. 5) and by indirect immunofluorescence (Table III).

Labeled proteins of the expected size (~80 kD for the glycosylated molecules or 60 kD for the nonglycosylated molecules) could be immunoprecipitated from extracts of cells expressing the wild-type and mutant HAs using monoclonal antisera specific for the A, B, or C sites as well as by the polyclonal anti-X31 HA serum (Fig. 5). Unfortunately, the affinity of the site D antibody appeared to be insufficient for immunoprecipitation of HA under the conditions we used, although it was suitable for use in immunofluorescence experiments (see below). The majority of the mutants were recognized by all three A, B, and C mAbs. However, mutant 54B+ was only weakly recognized by the C mAb, whether or not it was glycosylated. It is likely that this altered binding affinity may be due to the substitution of proline 55 by glycine. By contrast, the B mAb immunoprecipitated the nonglycosylated form of mutant 188+ although it recognized the glycosylated protein only weakly at 37°C. This result indicates that residue 190 does not form part of the B antibody

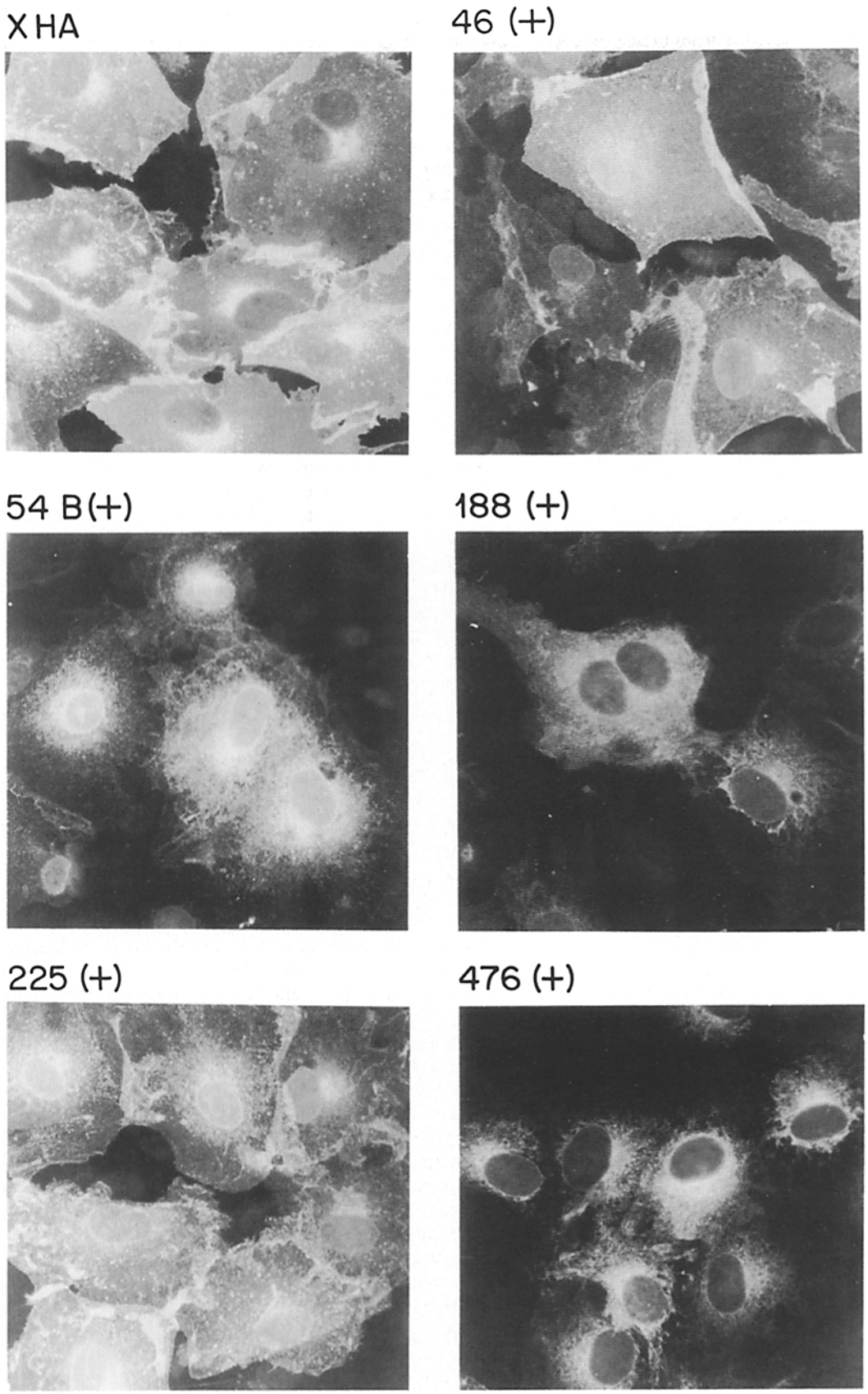

Figure 4. Intracellular localization of wild-type and mutant XHA proteins expressed in CV-1 cells during growth at 37°C. 40 h after infection with SV40–HA recombinant viruses, CV-1 cells were permeabilized and assayed by indirect immunofluorescence using a specific anti–X31 HA polyclonal rabbit serum as described by Doyle et al. (1985).

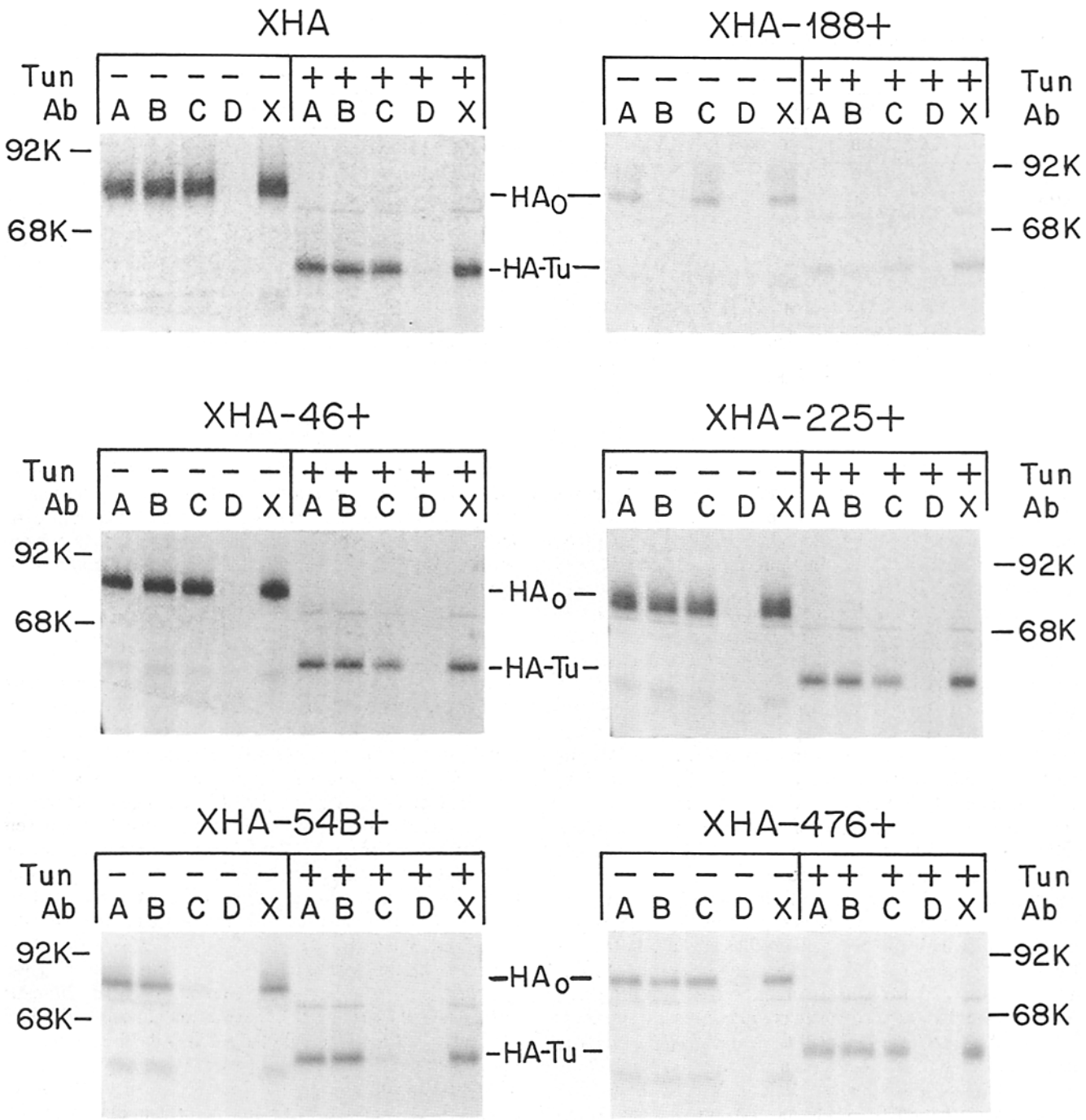

Figure 5. Recognition of wild-type and mutant XHA proteins by monoclonal antisera specific for the A, B, C, or D antigenic epitopes. CV-1 cells infected for 40 h with SV40–HA recombinant viruses were labeled in the presence or absence of tunicamycin (5 μg/ml) for 1 h with [^{35}S]methionine. Cell extracts were prepared, and proteins were immunoprecipitated with one of four mouse mAbs directed against the A, B, C, or D antigenic sites on XHA. The precipitated proteins were analyzed by SDS-PAGE and autoradiographed. The mAbs used were the following: 11/4 (*A*) 17/2 (*B*), 69/1 (*C*), and 78/6 (*D*). *X* is a specific rabbit anti-XHA polyclonal antiserum.

combining site since the substitution of Glu 190 by Ser to form the Asn-Gln-Ser consensus sequence did not prevent antibody binding in the absence of glycosylation. The weak recognition of the glycosylated protein could result either from shielding of the region by the oligosaccharide side chain or from modification of the ε-amino group of Asn_{188}. Substitution of this residue by aspartic acid has been observed in virus variants that escape neutralization by the B antibody (Wiley and Skehel, 1987).

Indirect immunofluorescence of permeabilized cells could be used to assay recognition of the wild-type and mutant HAs by the complete panel of mAbs since the D antibody recognized HA in immunofluorescence experiments. The results obtained are summarized in Table III. mAb A recognized the

Table III. Effect of Temperature on the Antigenic Epitopes and Functional Activities of Wild-type and Mutant HA Glycoproteins

	Assay results at 37°C						Assay results at 30°C					
	Binding to mAbs				RBC binding	Cell/cell fusion	Binding to mAbs				RBC binding	Cell/cell fusion
	A	B	C	D			A	B	C	D		
XHA	+	+	+	+	>90%	+++	+	+	+	+	>70%	+++
XHA46+	+	+	+	+	>90%	+++	+	+	+	+	>70%	+++
XHA54A+	+	+	+	−	>90%	+++	+	+	+	+	>70%	+++
XHA54B+	+	+	±*	−	>90%	+++	+	+	±	+	>70%	+++
XHA188+	+	±	+	−	0%	+++	+	+	+	+	5%	+++
XHA225+	+	+	+	+	>90%	+++	+	+	+	+	>70%	+++
XHA476+	+	+	+	−	0%	−	+	+	+	+	>70%	+++

The synthesis of HA glycoproteins at 37 or 30°C in CV-1 cells infected with SV40-HA recombinant viruses and the subsequent immunofluorescence, erythrocyte (*RBC*) binding, and cell/cell fusion assays were carried out as described in Materials and Methods.
* ± indicates weak recognition by the antibody.

wild-type HA and all the mutant proteins, revealing fluorescent-staining patterns similar to those seen with the polyclonal antiserum (see Fig. 4). The patterns of recognition of the mutant proteins by the A, B, and C mAbs were consistent with those obtained using immunoprecipitation (Fig. 5). After synthesis at 37°C, only cells expressing the wild-type HA or mutants 46+ and 225+ displayed surface and internal fluorescence with mAb D. However, when the proteins were synthesized and accumulated at 30°C, every mutant HA could be recognized by the D antibody, yielding an intense cell surface and internal staining pattern. Since the D antibody appears to recognize an epitope that is formed solely when the globular domains at the top of the molecules are packed together correctly (Wiley et al., 1981), it seems that only mutants 46+ and 225+ display the native trimeric structure at 37°C. However, all of the mutant proteins appear to be correctly folded and oligomerized at 30°C since all were recognized by the D antibody.

Conformational changes in HA have been shown to enhance its suceptibility to proteases, presumably by exposure of protease-sensitive sites which are inaccessible in the native protein (Skehel et al., 1982; Doms and Helenius, 1986). We have shown previously that monomeric or improperly folded forms of HA are degraded by trypsin, while correctly folded HA trimers are resistant to degradation by the protease although the HA molecules are cleaved into their component HA1 and HA2 chains (Gething et al., 1986*b*). The protease sensitivity of the wild-type and mutant proteins was determined by preparing cell extracts from infected, labeled cells in the absence of protease inhibitors and then incubating the extracts with trypsin (25 μg/ml) for 15 min at 4°C. After the addition of an excess of soybean trypsin inhibitor, the HA polypeptides in the cell extracts were immunoprecipitated and separated by SDS-PAGE (Fig. 6 *A*). Mutant 476+ was completely degraded by trypsin, suggesting that its structure is unfolded after synthesis at 37°C. By contrast, wild-type HA and the other mutants were cleaved into HA1 and HA2 subunits that were completely or largely resistant to further digestion by the protease at 4°C (Fig. 6 *A*). As observed in previous experiments (Figs. 3 *A* and 4), mutant 188+ displayed increased susceptibility to degradation by trypsin, although significant amounts of intact HA1 and HA2 subunits remained after digestion of the cell extract at 4°C.

After synthesis at 30°C, all the mutant proteins, including 476+ (Fig. 6 *B*), were resistant to degradation by trypsin although they could be cleaved into stable HA1 and HA2 subunits. These results are consistent with all of the mutants becoming folded into the correct trimeric structure at the lower temperature.

Functional Activities of Wild-type and Mutant HAs

Functional HA displayed on the cell surface can be detected using two assays which reflect the receptor-binding and membrane-fusion activities of the molecules that are involved in the infectious entry of influenza virions into the host cell (for a review see Gething and Sambrook, 1983). The sialic acid–binding activity of HA can be monitored by hemagglutination of erythrocytes to infected cell monolayers (Gething and Sambrook, 1981). Table III summarizes the results obtained when cells infected for 40 h with the various recombinant viruses were assayed for erythrocyte binding. At 37°C, >90% of the cells which expressed either the wild-type HA or the 46+, 54A+, 54B+, and 225+ HA mutants bound erythrocytes densely. However, cells expressing two of the mutants (188+ and 476+) failed to bind erythrocytes after synthesis at 37°C. In the case of mutant 476+, this lack of binding is almost certainly the result of the failure to transport functional HA to the cell surface since expression at 30°C resulted in binding of erythrocytes to >70% of the cells. Lack of transport of the mutant protein cannot provide the explanation for the failure of cells expressing mutant 188+ to bind significant numbers of erythrocytes, because adequate levels of functional protein are present at the cell surface after synthesis at either temperature (see Fig. 3 and results of the fusion assays discussed below). It is likely that the supernumerary oligosaccharide masks the activity of the sialic acid receptor either by shielding or disrupting the binding site.

We have shown previously that CV-1 cells expressing HA on their plasma membranes can be induced to fuse with each other after brief exposure to low pH (White et al., 1982; Gething et al., 1986*a*). This results in the formation of large syncytial cells and occurs within a defined pH range. To analyze the ability of the wild-type and mutant HAs to mediate low pH–induced fusion, infected CV-1 cells were first treated

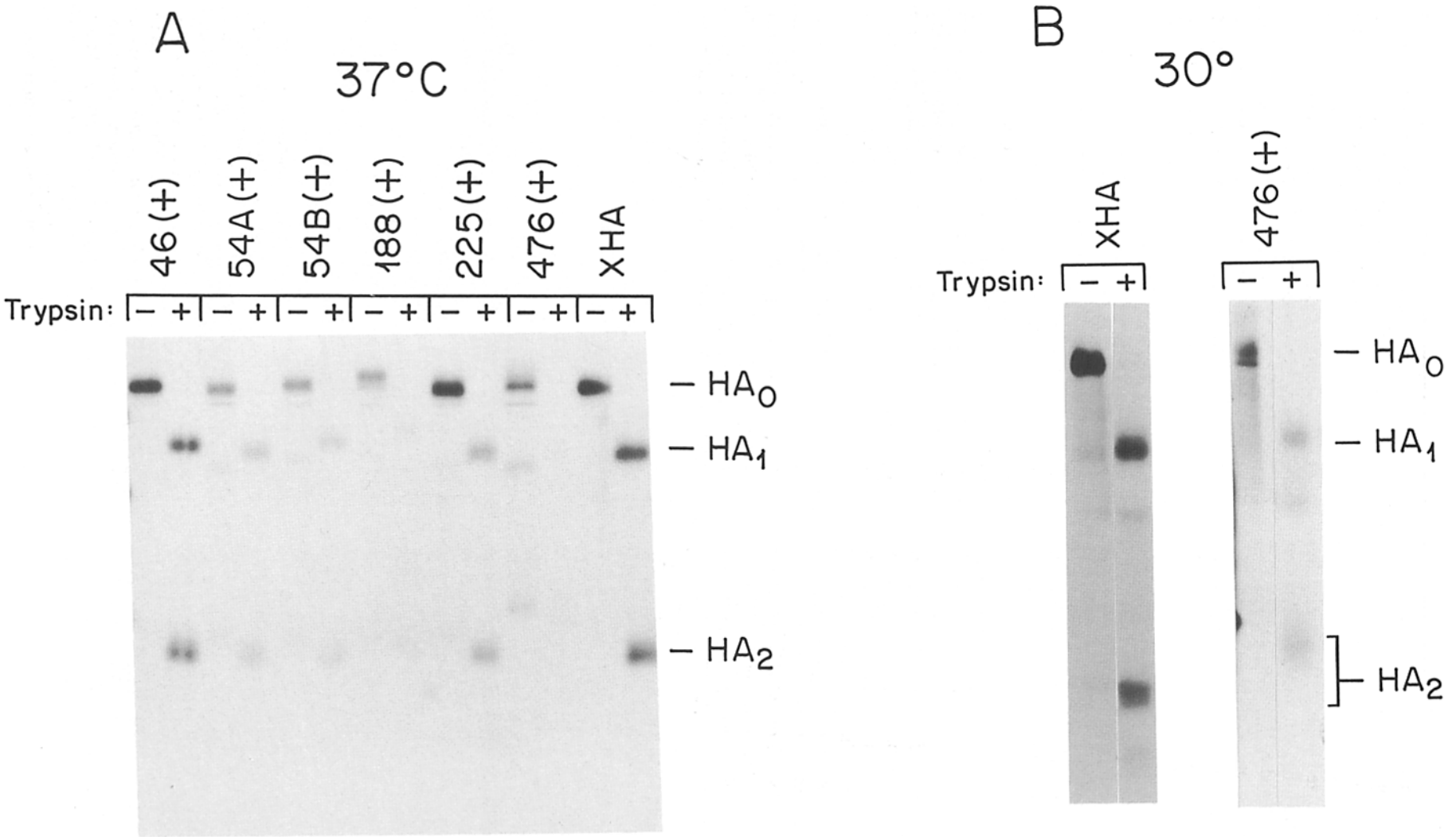

Figure 6. Protease sensitivity of wild-type and mutant XHA proteins. CV-1 cells infected for 40 h with SV40–HA recombinant viruses were labeled for 1 h at 37 (*A*) or 30°C (*B*) with [^{35}S]methionine. Cell extracts were prepared in the absence of protease inhibitors and then incubated for 15 min at 4°C with trypsin (25 μg/ml). After the addition of soybean trypsin inhibitor to 100 μg/ml, HA polypeptides were immunoprecipitated, separated by SDS-PAGE, and autoradiographed.

with trypsin to convert the precursor HA0 to the active form (Gething and Sambrook, 1982). The pH of the medium was then transiently decreased to values between 5 and 6 before incubation of the cells in normal medium for several hours to facilitate visualization of the formation of polykaryons. The results of these experiments and others performed after synthesis at 30°C are summarized in Table III. After synthesis at 37°C, only mutant 476+ failed to mediate cell fusion within a pH range between 5 and 6. However, when synthesized at 30°C, all the mutant proteins could mediate the fusion reaction with the same pH dependence as that displayed by the wild-type protein. The failure to transport a sufficient amount of functional HA to the cell surface at 37°C (see above and Fig. 3 *A*) provides the explanation for the lack of fusion of cells expressing mutant 476+. More interestingly, the ability of mutant 188+ to mediate cell fusion despite its lack of hemagglutination activity demonstrates that binding of HA to sialic acid–containing residues on the target membrane is not a prerequisite for membrane fusion leading to polykaryon formation.

Discussion

We have constructed and expressed a series of mutant HA molecules, each containing a new consensus site for glycosylation in addition to the seven sites normally found on the wild-type protein. Knowledge of the precise three-dimensional structure of the X-31 HA molecule and of the amino acid sequences of a large number of other HAs from different virus strains allowed us to choose novel sites for glycosylation which were located within five areas on the protein's surface that have not previously been shielded by an oligosaccharide side chain. We were successful in creating functional consensus sequences for glycosylation at four of the five sites that were mutated. The effects of the supernumerary oligosaccharides on the structure, intracellular transport, and biological activities of HA are summarized in Fig. 7. The results suggest that there are at least two reasons for the evolutionary exclusion of carbohydrate from specific areas of the surface of the HA molecule; attachment of an oligosaccharide at some positions can (*a*) interfere with the folding and assembly of the HA molecule and/or (*b*) shield or disrupt a functional region on the surface of the protein. However, attachment of an oligosaccharide side chain at one position (residue 46) had no discernible effect on the structure or function of HA. It is possible that analysis of additional natural HA variants might reveal one that contains carbohydrate attached at or near this site. Alternatively, the presence of carbohydrate at this position on the molecule may have been selected against during evolution for a reason that we have not been able to assay in the tissue culture system used for this work, such as a defect in directional transport or virus assembly. This possibility is currently under investigation.

We have previously shown that correct folding and trimerization of HA is a prerequisite for efficient mobilization of the protein into the secretory pathway and that unfolded or partially folded forms of HA are unable to leave the ER (Gething et al., 1986*b*). In this study we again observe that transport defects correlate with aberrant folding of the HA molecule. The only mutants that move to the Golgi apparatus with kinetics similar to the wild-type protein are those (46+ and 225+) that achieve a protease-resistant conformation and display the D antigenic epitope characteristic of the trimeric, wild-type structure. Those mutants (54A+, 54B+, and 188+) that display partial, temperature-sensitive defects in transport show parallel defects in folding as measured by for-

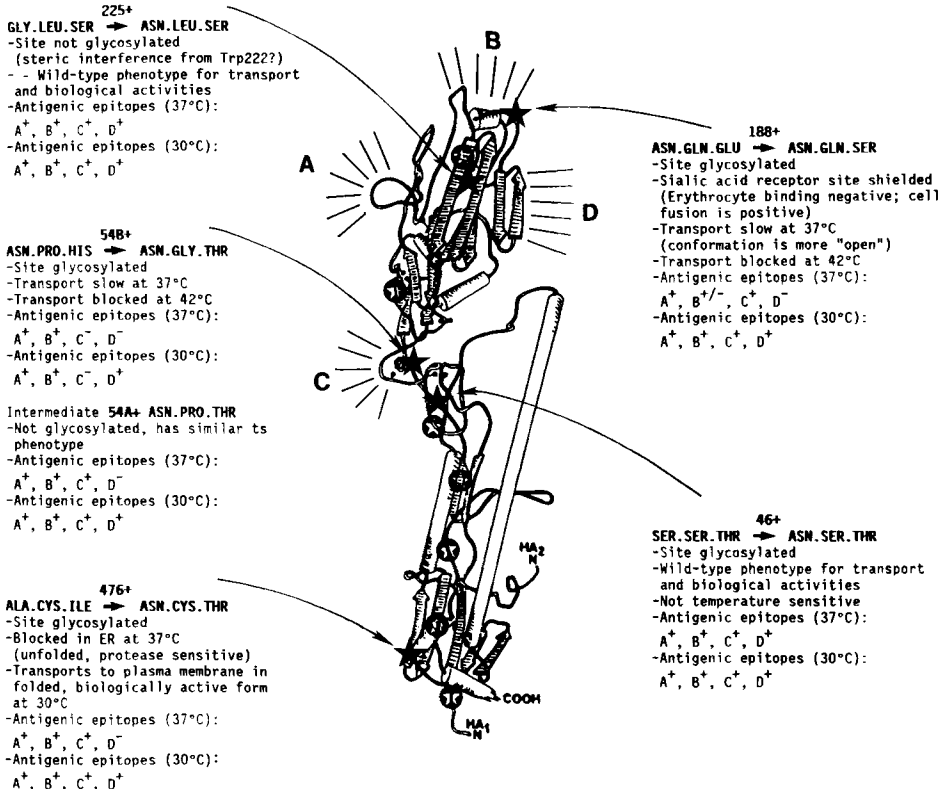

Figure 7. Summary of the phenotypes of mutants having supernumerary consensus sites for glycosylation. A drawing of the ectodomain of an HA monomer (Wilson et al., 1981) shows the positions of the seven natural (*open stars within circles*) and five supernumerary glycosylation sequences (*solid stars*). The positions of the four major antigenic epitopes (*A*, *B*, *C*, and *D*; Wiley et al., 1981) are also shown.

mation of the D antigenic epitope. Finally, the defective transport of mutant 476+ from the ER after synthesis at 37°C correlates with the protein remaining in an unfolded, protease-sensitive conformation. Evidence from cross-linking studies suggests that 476+ does not assemble into trimers at 37°C (our unpublished results). In the absence of structural information about this mutant, it would have been tempting to surmise that the oligosaccharide side chain attached to residue 476 inhibited the transport of the protein by shielding an epitope involved in exocytosis. However, the ability of the mutant to fold correctly and move normally along the secretory pathway after synthesis at 30°C disproves any role for shielding and confirms the importance of folding in facilitating transport from the ER.

In experiments aimed at determining whether oligosaccharides play a direct role in promoting protein transport to the cell surface, Machamer and Rose (1988*a*, *b*) have used oligonucleotide-directed mutagenesis to introduce novel glycosylation sites into the G protein of vesicular stomatitis virus. Because the three-dimensional structure of G is not known, these new sites were located in regions predicted by computer algorithms to be exposed in the folded molecule. Oligosaccharides added at two new positions on the polypeptide chain could promote the cell surface expression of a G protein lacking carbohydrate at the two normal sites, although the intracellular transport of these proteins was temperature-sensitive. Oligosaccharides added at four other positions on the polypeptide backbone did not promote the transport of the mutant G protein and in many cases interfered with the transport of G that is glycosylated at the two normal sites. It was concluded that the primary role of the natural oligosaccharides in the intracellular transport of G protein lies in promoting proper polypeptide folding, as previously suggested by Gibson et al. (1979). We have reached a similar conclusion for HA by using oligonucleotide-directed mutagenesis to remove the seven natural glycosylation sites in the X31-HA polypeptide chain, both singly and in combination (Gallagher, P., J. Henneberry, J. Sambrook, and M.-J. Gething, manuscript in preparation). Taken together with the results reported in this paper, these studies show that folding defects in glycoproteins can result either from the subtraction of natural oligosaccharide side chains or from the addition of supernumerary ones. Clearly, however, not all alterations in the folding or stability of these types of mutant glycoproteins are attributable solely to the presence or absence of an oligosaccharide side chain (Santos-Aguardo et al., 1987). The amino acid substitution(s) introduced to construct the novel consensus sites could by themselves affect the protein structure, despite care having been taken in this study to target residues in HA that had not been conserved during evolution. It is not possible for us to apportion the contribution of either the oligosaccharide side chain or the amino acid change(s) in HA to any folding defects because the nonglycosylated XHA molecules synthesized in the presence of tunicamycin are not assembled into native trimers or transported from the ER (our unpublished results). However, it is apparent that the amino acid substitution of threonine for histidine in the 54A+ mutant is responsible for the observed folding and transport defects, since the proline residue in the center of the consensus sequence prevents addition of a supernumerary oligosaccharide chain.

The long term goal of this study is to use supernumerary oligosaccharides to search for the location of functional epitopes on the HA molecule. The ability of carbohydrate to shield surfaces of HA from the immune system has been demonstrated in nature during the antigenic evolution of influenza viruses (reviewed by Wiley and Skehel, 1987). Selective pressure during evolution must have allowed the emergence only of those viruses containing mutated HA molecules that can be efficiently incorporated into virions while maintaining function and thermostability. Thus evolution limits the positions of novel glycosylation consensus sequences to those where oligosaccharide addition has minimal effects on the folding, transport, and activity of the HA molecule. In our in vitro experiments with HA, it was not possible to use genetic selection to reveal the least disruptive locations for supernumerary glycosylation sites and choices had to be made based upon available knowledge of the sequence and structure of the protein. However, we were able to select with high efficiency sites at which attachment of carbohydrate, under the appropriate permissive conditions, did not prevent the transport of HA to the plasma membrane where it could be assayed for the display of functional epitopes. Accurate pinpointing of new epitopes on HA depends upon the supernumerary oligosaccharides having only local effects on the structure of the molecule. The feasibility of the method is borne out by our observations (Table III and Fig. 7) that after synthesis of the mutants at the permissive temperature of 30°C, none of the supernumerary oligosaccharides had any effect on the recognition of antigenic epitopes or the function of biologically active sites that lay at a distance from the novel carbohydrate.

This demonstration that in vitro mutagenesis can be used to produce transport-competent HA molecules containing supernumerary oligosaccharides that mask functional epitopes raises the possibility that the method can be used as a general tool for shielding chosen areas of the surface of proteins that enter or traverse the secretory pathway. If assays are available to rule out effects of global conformational changes, this method can be used either to pinpoint the location of functional epitopes on the surface of a protein or to mask the activity of a previously identified functional site. In both cases, the advantage of introducing a point mutation that leads to the addition of an oligosaccharide over one that causes only a single amino acid change lies in the increase in the area of the protein whose function can be probed since an oligosaccharide side chain can shield an area covering a number of amino acids. In a search for a functional epitope, the number of point mutants that would need to be constructed to probe the protein surface would be too large for the approach to be feasible in the absence of a genetic screen. However, many fewer oligosaccharide-addition mutants would suffice to survey the entire surface of a protein.

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References

Air, G. M., W. G. Laver, and R. G. Webster. 1987. Antigenic variation in influenza viruses. Antigenic variation: molecular and genetic mechanisms of relapsing disease. *Contrib. Microbiol. Immunol.* 8:22–59.

Ash, J. F., D. Louvard, and S. J. Singer. 1977. Antibody-induced linkages of plasma membrane proteins to intracellular actomyosin-containing filaments in cultured fibroblasts. *Proc. Natl. Acad. Sci. USA.* 74:5584–5588.

Bause, E. 1983. Structural requirements of *N*-glycosylation of proteins. *Biochem. J.* 209:331–336.

Blobel, G. 1980. Intracellular protein topogenesis. *Proc. Natl. Acad. Sci. USA.* 77:1496–1500.

Bole, D. G., L. M. Hendershot, and J. F. Kearney. 1986. Posttranslational association of immunoglobulin heavy chains in nonsecreting and secreting hybridomas. *J. Cell Biol.* 102:1558–1566.

Caton, A. J., G. G. Brownlee, J. W. Yewdell, and W. Gerhard. 1982. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). *Cell.* 31:417–427.

Connolly, M. L., and A. R. Olson. 1985. Granny: a companion to Gramps for the real-time manipulation of macromolecular models. *Comput. & Chem.* 9: 1–6.

Doms, R. W., and A. Helenius. 1986. Quaternary structure of influenza virus hemagglutinin after acid treatment. *J. Virol.* 60:833–839.

Doyle, C., M. G. Roth, J. Sambrook, and M.-J. Gething. 1985. Mutations in the cytoplasmic domain of the influenza virus hemagglutinin affect different stages of intracellular transport. *J. Cell Biol.* 100:704–714.

Doyle, C., J. Sambrook, and M.-J. Gething. 1986. Analysis of progressive deletions of the transmembrane and cytoplasmic domains of influenza hemagglutinin. *J. Cell Biol.* 103:1193–1204.

Elbein, A. D. 1981. The tunicamycins: useful tools for studies on glycoproteins. *Trends Biochem. Sci.* 6:219–221.

Elbein, A. D. 1987. Inhibitors of the biosynthesis and processing of N-linked oligosaccharide chains. *Annu. Rev. Biochem.* 56:497–534.

Fitting, T., and D. Kabat. 1982. Evidence for a glycoprotein "signal" involved in transport between subcellular organelles. *J. Biol. Chem.* 257:14011–14017.

Gething, M.-J., and J. Sambrook. 1981. Cell surface expression of influenza haemagglutinin from a cloned DNA copy of the RNA gene. *Nature (Lond.).* 293:620–625.

Gething, M.-J., and J. Sambrook. 1982. Construction of influenza haemagglutinin genes that code for intracellular and secreted forms of the protein. *Nature (Lond.).* 300:598–603.

Gething, M.-J., and J. Sambrook. 1983. Expression of cloned influenza virus genes. *In* Genetics of Influenza Viruses. P. Palese and D. Kingsbury, editors. Springer-Verlag, Berlin. 169–191.

Gething, M.-J., R. W. Doms, D. York, and J. White. 1986*a*. Studies on the mechanism of membrane fusion: site-specific mutagenesis of the hemagglutinin of influenza virus. *J. Cell Biol.* 102:11–23.

Gething, M.-J., K. McCammon, and J. Sambrook. 1986*b*. Expression of wild-type and mutant forms of influenza hemagglutinin: the role of folding in intracellular transport. *Cell.* 46:939–950.

Gibson, R., S. Kornfeld, and S. Schlesinger. 1980. A role for oligosaccharides in glycoprotein biosynthesis. *Trends Biol. Sci.* 5:290–293.

Gibson, R., S. Schlesinger, and S. Kornfeld. 1979. The non-glycosylated glycoprotein of vesicular stomatitis virus is temperature-sensitive and undergoes intracellular aggregation at elevated temperatures. *J. Biol. Chem.* 254: 3600–3607.

Guan, J.-L., H. Cao, and J. K. Rose. 1988. Cell-surface expression of a membrane-anchored form of the human chorionic gonadotropin alpha-subunit. *J. Biol. Chem.* 263:5306–5313.

Guan, J.-L., C. E. Machamer, and J. K. Rose. 1985. Glycosylation allows cell-surface transport of an anchored secretory protein. *Cell.* 42:489–496.

Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* 166:557–580.

Keil, W., H. Niemann, R. T. Schwarz, and H.-D. Klenk. 1984. Carbohydrates of influenza virus III. Nature of oligosaccharide-protein linkage in viral glycoproteins. *Virology.* 133:77–91.

Klenk, H.-D. 1980. Processing of the hemagglutinin: glycosylation and proteolytic cleavage. *In* Structure and Variation in Influenza Virus. G. Laver and G. Air, editors. Elsevier Science Publishing Co., Inc., New York. 213–222.

Kornfeld, R., and S. Kornfeld. 1985. Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* 54:631–664.

Machamer, C. E., and J. K. Rose. 1988*a*. Influence of new glycosylation sites on expression of the vesicular stomatitis virus G protein at the plasma membrane. *J. Biol. Chem.* 263:5948–5954.

Machamer, C. E., and J. K. Rose. 1988*b*. Vesicular stomatitis virus G proteins display temperature-sensitive intracellular transport and are subject to aberrant intermolecular disulfide bonding. *J. Biol. Chem.* 263:5955–5960.

Machamer, C. E., R. Z. Florkiewicz, and J. K. Rose. 1985. A single N-linked oligosaccharide at either of the two normal sites is sufficient for transport of vesicular stomatitis virus G protein to the cell surface. *Mol. Cell. Biol.* 5: 3074–3083.

Matzuk, M. M., and I. Boime. 1988. The role of the asparagine-linked oligosaccharides of the alpha subunit in the secretion and assembly of human chorionic gonadotropin. *J. Cell. Biol.* 106:1049–1059.

Miyazaki, J.-I., E. Appella, H. Zhao, J. Forman, and K. Ozato. 1986. Expression and function of a nonglycosylated major histocompatibility class I antigen. *J. Exp. Med.* 163:856–871.

Nakamura, K., and R. W. Compans. 1979. Host cell- and virus strain-dependent differences in oligosaccharides of hemagglutinin glycoproteins of influenza A viruses. *Virology.* 95:8–23.

Neuberger, A., A. Gottschalk, R. O. Marshall, and R. Spiro. 1972. Carbohydrate-peptide linkages in glycoproteins and methods for their elucidation. *In* The Glycoproteins: Their Composition, Structure and Function. A. Gottschalk, editor. Elsevier Scientific Publishing Co., Amsterdam. 450–490.

O'Donnell, T. J., and A. J. Olson. 1981. Gramps, an interactive graphics language for widening chemical graphics applications. *Computer Graphics.* 15: 133–142.

Olden, K., B. A. Bernard, M. J. Humphries, T.-K. Yeo, K.-T. Yeo, S. L. White, S. A. Newton, H. C. Bauer, and J. B. Parent. 1985. Function of glycoprotein glycans. *Trends Biochem. Sci.* 10:78–82.

Owen, M. J., A.-M. Kissonerghis, and H. F. Lodish. 1980. Biosynthesis of the HLA-A and HLA-B antigens in vivo. *J. Biol. Chem.* 255:9678–9684.

Poruchynsky, M. S., C. Tyndall, G. W. Both, F. Sato, A. R. Bellamy, and P. H. Atkinson. 1985. Deletions into an NH_2-terminal hydrophobic domain result in secretion of rotavirus VP7, a resident endoplasmic reticulum membrane glycoprotein. *J. Cell Biol.* 101:2199–2209.

Raymond, F. L., A. J. Caton, N. J. Cox, A. P. Kendal, and G. G. Brownlee. 1986. The antigenicity and evolution of influenza H1 haemaglutinin, from 1950–1957 and 1977–1983: two pathways from one gene. *Virology.* 148: 275–287.

Rodriguez-Boulan, E., and D. Sabatini. 1978. Polarized distribution of viral envelope proteins in the plasma membrane of infected epithelial cells. *Cell.* 20:45–55.

Sambrook, J., T. Maniatis, and E. Fritsch. 1988. *Molecular Cloning: A Laboratory Manual* 2nd Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. 545 pp.

Santos-Aguado, J., P. A. Biro, U. Fuhrmann, J. L. Strominger, and J. A. Barbosa. 1987. Amino acid sequences in the alpha-1 domain and not glycosylation are important in HLA-A2/B2-microglobulin association and cell surface expression. *Mol. Cell. Biol.* 7:982–990.

Schuy, W., C. Will, K. Kuroda, C. Schlotissek, W. Garten, and H.-D. Klenk. 1986. Mutations blocking the transport of the influenza virus hemagglutinin between the rough endoplasmic reticulum and the Golgi apparatus. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:2831–2836.

Schwarz, R. T., and R. Datema. 1980. Inhibitors of protein glycosylation. *Trends Biochem. Sci.* 5:65–67.

Skehel, J. J., P. M. Bayley, E. B. Brown, S. R. Martin, M. D. Waterfield, J. M. White, I. A. Wilson, and D. C. Wiley. 1982. Changes in the conformation of influenza virus hemagglutinin at the pH optimum of virus-mediated membrane fusion. *Proc. Natl. Acad. Sci. USA.* 79:968–972.

Skehel, J. J., D. J. Stevens, R. S. Daniels, A. R. Douglas, M. Knossow, I. A. Wilson, and D. C. Wiley. 1984. A carbohydrate side chain on hemagglutinins of Hong Kong influenza viruses inhibits recognition by a monoclonal antibody. *Proc. Natl. Acad. Sci. USA.* 81:1779–1783.

Webster, R. G., L. E. Brown, and D. C. Jackson. 1983. Changes in the antigenicity of the hemagglutinin molecule of H3 influenza viruses at acidic pH. *Virology.* 126:587–599.

White, J., A. Helenius, and M.-J. Gething. 1982. Haemagglutinin of influenza virus expressed from a cloned gene promotes membrane fusion. *Nature (Lond.).* 300:658–659.

Wiley, D. C., and J. J. Skehel. 1987. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. *Annu. Rev. Biochem.* 56: 365–394.

Wiley, D. C., I. A. Wilson, and J. J. Skehel. 1981. Structural identification of the antibody-binding sites of Hong Kong influenza virus haemagglutinin and their involvement in antigenic variation. *Nature (Lond.).* 289:373–378.

Wilson, I. A., R. C. Ladner, J. J. Skehel, and D. C. Wiley. 1983. The structure and role of the carbohydrate moieties of influenza virus hemagglutinin. *Biochem. Soc. Trans.* 11:145–147.

Wilson, I. A., J. J. Skehel, and D. C. Wiley. 1981. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 A resolution. *Nature (Lond.).* 289:366–373.

Zoller, M., and M. Smith. 1983. Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors. *Methods Enzymol.* 100:468–500.

Zoller, M., and M. Smith. 1985. Oligonucleotide-directed mutagenesis: a simple method using two oligonucleotide primers and a single-stranded DNA template. *DNA (NY).* 3:479–488.