

Cell Surface Expression of Glycosylated, Nonglycosylated, and Truncated Forms of a Cytoplasmic Protein Pyruvate Kinase

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Abstract. The soluble cytoplasmic protein pyruvate kinase (PK) has been expressed at the cell surface in a membrane-anchored form (APK). The hybrid protein contains the NH₂-terminal signal/anchor domain of a class II integral membrane protein (hemagglutinin/neuraminidase, of the paramyxovirus SV5) fused to the PK NH₂ terminus. APK contains a cryptic site that is used for N-linked glycosylation but elimination of this site by site-specific mutagenesis does not prevent cell

surface localization. Truncated forms of the APK molecule, with up to 80% of the PK region of APK removed, can also be expressed at the cell surface. These data suggest that neither the complete PK molecule nor its glycosylation are necessary for intracellular transport of PK to the cell surface, and it is possible that specific signals may not be needed in the ectodomain of this hybrid protein to specify cell surface localization.

THE signals for the partitioning of proteins into the lumen of the endoplasmic reticulum have been elucidated in some detail (Blobel and Dobberstein, 1975; Blobel, 1980; Walter and Blobel, 1981). Through the use of hybrid or chimeric molecules it has been demonstrated that a hydrophobic signal sequence at the NH₂ terminus of a protein is both necessary and sufficient to coordinate the translocation of the protein into the lumen of the endoplasmic reticulum (ER)¹ (e.g., Gething and Sambrook, 1982; Sveda et al., 1982; Rose and Bergman, 1982; Yost et al., 1983). However, less progress has been made in determining the signals necessary for transport beyond the ER to the Golgi apparatus and the cell surface, in the large part due to problems encountered in constructing chimeric proteins that can be transported beyond the Golgi apparatus.

There are likely to be many factors involved in the transport of proteins from the ER to the cell surface and each may influence the transportation of hybrid molecules. One factor which has been suggested to be important is the correct conformation and oligomerization of the protein. The oligomeric form may be a homo-oligomer as in the case of influenza virus hemagglutinin trimers (Gething et al., 1986; Copeland et al., 1986) and vesicular stomatitis virus (VSV) G glycoprotein (Kreis and Lodish, 1986), or a hetero-oligomeric form such as the binding of ligand by the retinol-binding protein before export (Ronne et al., 1983) and the interactions of the heavy chain of IgM with light chain (Mains and Sibley, 1983). A second possible factor that may influence transport is the addition of asparagine N-linked carbo-

hydrate at the consensus amino acid sequence Asn-X-Ser/Thr (reviewed by Kornfeld and Kornfeld, 1985). It has been suggested that glycosylation could act in a fashion analogous to the mannose-6-phosphate signal for lysosomal sorting (see von Figura and Hasilik, 1986, for review) as has been suggested (Guan et al., 1985; Machamer et al., 1985). Alternatively, the oligosaccharides could aid in the formation of the mature tertiary structure or in the oligomerization process. A third possible factor necessary for transport is that the protein encodes a positive acting signal either in its primary structure or in the form of a "signal patch" (conformational epitope) produced during the tertiary folding of the molecule (Pfeffer and Rothman, 1987).

The construction of chimeric proteins to define the roles of signal regions and membrane anchorage domains in the translocation process have mostly involved class I glycoproteins (reviewed in Garoff, 1985), i.e., those proteins with an NH₂-terminal signal domain that is usually cleaved and a COOH-terminal anchorage or stop-transfer domain (e.g., Yost et al., 1983; Guan and Rose, 1984; Guan et al., 1985). We have been interested in examining the requirements for cell surface expression of class II membrane proteins, i.e., those with a COOH-terminal ectodomain and an NH₂-terminal cytoplasmic domain. These proteins have a single uncleaved hydrophobic domain at the NH₂ terminus that has been shown to be sufficient and necessary to act as both a signal sequence and as a stop-transfer sequence (e.g., Bos et al., 1984).

The hemagglutinin/neuraminidase (HN) glycoprotein of the paramyxovirus SV5 is a prototype class II integral membrane protein having a single uncleaved NH₂-terminal hydrophobic domain that acts as an "extended signal/anchor" (Hiebert et al., 1985a). The HN protein contains all the necessary signals for intracellular transport to the cell sur-

1. *Abbreviations used in this paper:* APK, anchored pyruvate kinase; Endo-F and -H, endoglycosidase F and H, respectively; ER, endoplasmic reticulum; HN, hemagglutinin/neuraminidase; PK, pyruvate kinase; TM, tunicamycin.

face as demonstrated by the expression of biologically active HN at the cell surface from cloned cDNA (Paterson et al., 1985). To determine the importance of the NH₂-terminal domain of HN in cell surface localization and to determine whether possible transport signals are contained within the primary structure of a protein, we have constructed chimeric proteins containing the cytoplasmic tail and hydrophobic signal/anchor region of HN joined to the NH₂ terminus of pyruvate kinase (PK). It would not be expected that PK would contain transport signals for the exocytotic pathway as PK is a $M_r \sim 60,000$ cytoplasmic, soluble enzyme which forms homo-oligomeric tetramers and is not known to interact with membranes. We present evidence here that the NH₂-terminal domain of HN (signal/anchor) is both necessary and sufficient for targeting PK to the ER membrane and anchoring it in the ER in a stable manner, that the hybrid integral membrane protein is expressed at the cell surface, and that neither glycosylation nor the complete PK molecule are necessary for the cell surface expression of PK.

Materials and Methods

Plasmid Construction

The anchored membrane form (APK) (HN/PK) hybrid gene was constructed as follows. The HN cDNA insert was isolated from pSV103HN (Paterson et al., 1985) by Xho I digestion and the single Bgl I site in the HN cDNA was changed to Eco RI using Bgl I digestion, treatment with T₄ DNA polymerase, and ligation of Eco RI linkers. The PK cDNA clone pRL142PK10 (Lonberg and Gilbert, 1983; Kalderon et al., 1984; Richardson et al., 1986) was kindly provided by Drs. Bruce Roberts and Alan Smith, Integrated Genetics Inc., Framingham, MA. The Bgl II site at the 3' terminus of the PK cDNA was changed to an Xho I site by Bgl II digestion, treatment with T₄ DNA polymerase, and ligation of Xho I linkers. The APK hybrid gene was constructed by ligation of the small Xho I to Eco RI HN DNA fragment encoding the first 48 NH₂-terminal amino acids of HN to the large Eco RI to Xho I fragment which encodes PK residues 17 to 529 of the chicken pyruvate kinase cDNA. APK clones were selected that maintained the correct reading frame such that HN residues 1-48 were joined to PK residues 17 to 529, by sequencing plasmid DNA using the dideoxynucleotide chain-terminating method, and a synthetic oligonucleotide as primer (Paterson and Lamb, 1987). DNA subcloning steps were performed by inserting fragments directly into the SV-40 late region expression vector pSV63 which lacks the SV-40 late region from SV nucleotides 346-2,533 (Buchman et al., 1980). This vector was modified from pSV73 (Lamb and Lai, 1982) to contain a Xho I cloning site instead of Bam HI. As a control, the chicken PK gene was expressed in the pSV63 vector. The PK cDNA insert from pRL142PK10 (Kalderon et al., 1984) was isolated by Hind III and Bgl II digestion, treatment with T₄ DNA polymerase, addition of Xho I linkers, and ligation into pSV63. The encoded PK contains five amino acids at its NH₂ terminus including the initiating methionine codon, derived from the SV-40 early region and residues 17-529 of PK as described previously (Kalderon et al., 1984).

The glycosylation site Asn-Phe-Ser in APK (residues 74-76) was altered to Asn-Phe-Ala by site specific mutagenesis. APK DNA was subcloned into the replicative form of bacteriophage M13mp19X which is a derivative of M13mp19 containing an Xho I site in place of the Bam HI site in the poly-linker region (Paterson and Lamb, 1987). Single-stranded DNA was used as a template for oligonucleotide directed mutagenesis using the method of Zoller and Smith (1984). Mutants (APK*) were verified by dideoxynucleotide chain-terminating sequencing of the M13mp19XAPK* single-strand DNA (Sanger et al., 1977). The APK* mutant DNA insert was isolated from replicative form of M13mp19XAPK* and subcloned into the pSV63 vector. The nucleotide sequence of the entire APK* DNA was confirmed by the chemical method (Maxam and Gilbert, 1980).

The T₁ truncated form of APK was constructed by subcloning the 5'-terminal Xho I to Sal I (nucleotide 722 of PK sequence) DNA fragment of APK into pSV63. The encoded polypeptides contain in addition to PK residues, 10 COOH-terminal amino acids derived from SV-40 sequences before the first termination codon (see Fig. 1).

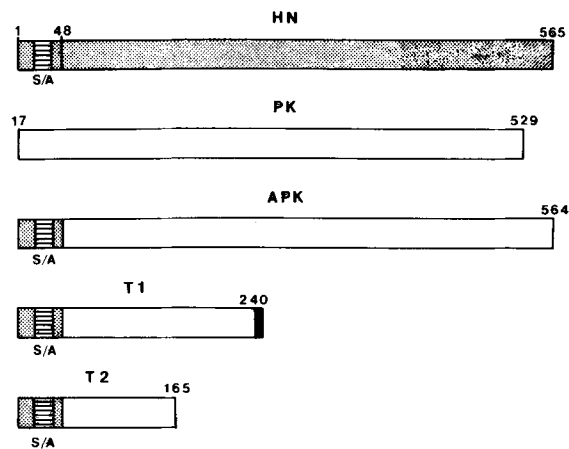


Figure 1. Construction of the APK hybrid protein and the T₁ and T₂ truncations of APK. The SV5 HN protein is shown schematically at the top. The NH₂-terminal domain including the cytoplasmic tail and the uncleaved signal/anchor is indicated as residues 1-48. S/A represents the single and uncleaved hydrophobic combined signal/membrane anchor domain of HN. Chicken PK is shown as an open box (residues 17-529). The hybrid protein, APK, was constructed by joining the NH₂-terminal domain of HN to residues 17-529 (open box) of PK through an Eco RI linker which resulted in the addition of 3 amino acids between the HN and PK sequences. The T₁ truncated form of APK was constructed by subcloning the small Xho I to Sal I restriction fragment of the APK molecule. The T₁ hybrid protein contains the NH₂-terminal 230 amino acids of APK and continues into SV-40 sequences for 10 codons (black box) yielding a 240 amino acid product. The T₂ truncation of APK was constructed by insertion of an oligonucleotide encoding stop codons in all three reading frames at the Sac I site of APK in the vector containing the T₁ truncation. These constructions were expressed in an SV-40 late region replacement vector, pSV63. Amino acid numbers are indicated above each construction.

The T₂ truncation of APK was constructed by subcloning into the Sac I site of PK (nucleotide 474) a synthetic double-stranded DNA fragment (5'-TTAGTAACTAG-3' and its complement), that contains stop codons in all three reading frames (Fig. 1). Oligonucleotides were synthesized by the Northwestern University Biotechnology facility on DNA synthesizer (model 380B; Applied Biosystems, Inc., Foster City, CA) and purified as described previously (Williams and Lamb, 1986; Paterson and Lamb, 1987).

Cells

Monolayer cultures of the TC7 clone of CV1 cells were grown in DME containing 10% FBS.

Preparation of Monospecific IgG

Antibody production in rabbits and affinity purification of anti-pyruvate kinase sera was done as described previously (Richardson et al., 1985). Chicken muscle pyruvate kinase type X (Sigma Chemical Co., St. Louis, MO) was used as antigen and 1.0 mg of PK was used per injection of which 0.5 mg was denatured by boiling in 1% SDS before the injection. Affinity purification of anti-PK sera was performed using PK conjugated to Affigel-10 resin (Bio-Rad Laboratories, Richmond, CA) and the antibodies were eluted in 0.1 M citrate buffer with 10% dioxane. Monospecific antisera to the HN protein was described previously (Paterson et al., 1985).

Transfection and Preparation of Recombinant Virus

The SV-40 DNA sequences were released from the pBR322 sequences in the vector by Xba I digestion followed by ligation at low DNA concentration to recircularize the SV-40 DNA sequences. Transfection of recombinant DNA molecules into CV1 cells were performed using DEAE-dextran as de-

scribed (Sompayrac and Danna, 1981) followed by a 2-min treatment of the cells with 10% dimethyl sulfoxide (Lopata et al., 1984; Paterson et al., 1985). An SV-40 early region deletion mutant (dl1055) (Pipas et al., 1983) was used to complement the late region replacement vector to produce lytic virus stocks.

Metabolic Labeling of Polypeptides, Immunoprecipitation, and SDS-PAGE

SV-40 recombinant-virus infected CV1 cells were labeled with [³⁵S]methionine or [³⁵S]TranS label (ICN Biomedicals Inc., Irvine, CA) as described (Lamb and Lai, 1982). For the labeling of oligosaccharides, SV-40 recombinant virus-infected cells were labeled with 250 μCi [³H]mannose in glucose-free MEM per 1-cm-diam well of CV1 cells. Immunoprecipitation was done as described by Anderson and Blobel (1983). Briefly, cells were lysed in 1% SDS, lysates boiled for 5 min, and diluted with 4 vol of dilution buffer before the addition of antisera. The lysates were incubated with PK antisera or HN antisera for 2–6 h at 4°C before addition of agarose beads conjugated with *Staphylococcus aureus* protein A (Boehringer Mannheim Diagnostics, Inc., Indianapolis, IN). Electrophoresis of samples on SDS-polyacrylamide gels (SDS-PAGE) was done as described (Lamb and Chopin, 1976).

Cell Surface Trypsinization

Confluent monolayers of CV1 cells (10-cm-diam plates) were infected with SV-40 recombinant viruses and at 44 h after infection the cells were labeled with [³⁵S]methionine for 15 min, the label removed, and the cells incubated for varying periods up to 3 h (chase) in DME. Cells were scraped from the plates with a silicon rubber policeman, resuspended in PBS and treated with 200 μg/ml tosylamide phenylethyl chloromethyl ketone-treated trypsin (Organon Teknika, Malvern, PA) for 30 min at 37°C as described (Lamb et al., 1985). The digestion was stopped by the addition of 1,000 U trypsin inhibitor (Sigma Chemical Co.) and the cells lysed by boiling after being made 1% in SDS.

Alkali Treatment of Microsomes

Microsomes were prepared essentially according to the method of Adams and Rose (1985) except that the infected cell homogenate was pelleted through a 0.7 ml 10% sucrose cushion for 40 min at 45,000 rpm at 4°C in a TLA-100 table top ultracentrifuge using a TLS-55 swinging bucket rotor (Beckman Instruments, Inc., Palo Alto, CA). Alkali treatment was done as described (Paterson and Lamb, 1987).

Indirect Immunofluorescence

Coverslips of SV-40 recombinant virus-infected CV1 cells were prepared for surface fluorescence by fixation in 0.1% freshly prepared paraformaldehyde in PBS for 5 min at room temperature. For internal staining, the cells were fixed in paraformaldehyde and permeabilized in 100% acetone for 2 min at –20°C. Antibody binding and mounting of coverslips was done as described (Dreyfuss et al., 1984; Paterson et al., 1985) using affinity-purified rabbit PK antisera.

Endoglycosidase Analysis

For endoglycosidase H (Endo-H) analysis, SV-40 recombinant virus-infected CV1 cells were pulse labeled with [³⁵S]methionine for 15 min and incubated in DME (chase) for varying periods. The cells were lysed in 1% SDS and immunoprecipitated with PK antisera as described above. Endo-H or endoglycosidase F (Endo-F) digestions were then done as described previously (Williams and Lamb, 1986).

Tunicamycin Treatment of Cells

CV1 cells infected with SV-40 recombinant viruses were treated with 5 μg/ml tunicamycin (Calbiochem-Behring Corp., La Jolla, CA) in DME for 30 min before, during, and after labeling with radioisotope.

Quantitation of Autoradiograms

Quantitation of autoradiograms was performed by scanning densitometry using a Pharmacia-LKB Gel Scan XL Laser Densitometer (Pharmacia Inc., Piscataway, NJ).

Results

Criteria for Selection of Pyruvate Kinase as a Reporter Protein

The selection of pyruvate kinase as a model protein for cell surface expression using the NH₂-terminal hydrophobic domain of the HN glycoprotein was based upon several considerations. (a) A reporter molecule was required that would not be expected to contain either positively acting transport signals or retention signals (Pfeffer and Rothman, 1987). Pyruvate kinase is a cytoplasmic, soluble, glycolytic enzyme and any transport signals present in pyruvate kinase are fortuitous. (b) The three-dimensional x-ray crystallographic structure of PK has been determined to 2.6 Å resolution (Stuart et al., 1979; Muirhead et al., 1986), which could aid in predicting alterations to PK. The determined structure shows that the molecule displays a protruding NH₂ terminus. Previously, residues encoding the SV-40 T antigen nuclear localization signals have been added to the NH₂ terminus of PK and the resulting molecule is redirected from the cytoplasm to the nucleus (Kalderon et al., 1984). We wished to explore the possibility of modifying the NH₂ terminus of PK to redirect it to the cell surface. (c) The amino acid sequence of chicken pyruvate kinase contains a single potential site for asparagine N-linked glycosylation (Asn-Phe-Ser, residues 74–76; Lonberg and Gilbert, 1983). The addition of oligosaccharides has been suggested to be important for the cell surface localization of some glycoproteins (Guan et al., 1985; Machamer et al., 1985). Therefore, we wished to determine if this site is used and what effect if any glycosylation may have on intracellular transport.

Structure of APK and APK*

A DNA molecule was constructed that could express a membrane anchored form of chicken pyruvate kinase (APK) such that the NH₂-terminal region of HN (48 amino acids) including the cytoplasmic tail and hydrophobic domain was fused in frame to the amino terminus of PK (Fig. 1, and see Materials and Methods). The construction resulted in three new amino acids being introduced in the chimeric polypeptide between HN residues 1–48 and PK residues 17–529 yielding a molecule of 564 amino acids. A DNA molecule lacking the glycosylation site at residues 74–76, designated APK*, was constructed in which the serine residue at position 76 in pyruvate kinase was changed to an alanine residue (see Materials and Methods). This change in the conserved sequence Asn-X-Ser/Thr has been previously demonstrated to prevent N-linked glycosylation (Machamer et al., 1985). The wild-type form of PK is the SV-40/PK fusion protein characterized previously (Kalderon et al., 1984) and it contains the first five amino acids of large T antigen of SV-40 fused to residues 17–529 of chicken pyruvate kinase.

Expression of APK and APK*

The recombinant cDNA clones encoding the PK, APK, and APK* proteins were placed under the control of the SV-40 late region promoter, transfected into CV1 cells together with the SV-40 early region mutant dl1055 (Pipas et al., 1983), and viral stocks were made. The proteins were immunoprecipitated from [³⁵S]methionine-labeled infected CV1 cells with anti-chicken pyruvate kinase monospecific IgG and

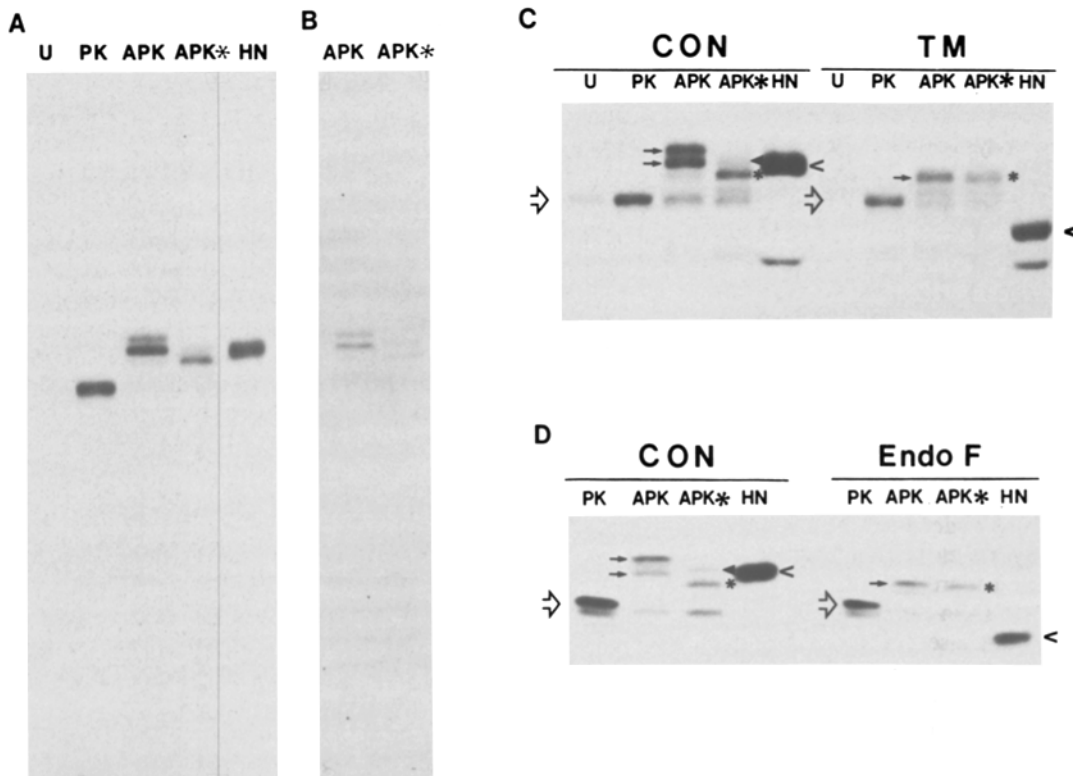


Figure 2. Expression of PK, APK, and APK* from SV-40 recombinant viruses, [³H]mannose labeling, sensitivity to treatment with Endo-F, and synthesis in the presence of TM. (A) Infected cells were labeled with [³⁵S]methionine, lysed in 1% SDS, immunoprecipitated with anti-chicken PK monospecific IgG, and analyzed on 8% polyacrylamide gels containing 4 M urea. Lane *U*, uninfected cells; lane *PK*, wild-type PK; lane *APK*, the APK hybrid protein; lane *APK**, the mutant lacking the N-linked glycosylation site at residues 74–76 of APK; lane *HN* shows similarly prepared labeled HN expressed from a SV-40 recombinant virus and immunoprecipitated with anti-HN monospecific IgG. (B) Infected cells were labeled with [³H]mannose, immunoprecipitated, and analyzed on gels as above. Lane *APK*, wild-type APK; lane *APK**, the mutant lacking the N-linked glycosylation site at residues 74–76. (C) SV-40 recombinant virus-infected cells were untreated (lanes marked *CON*) or treated with TM (lanes marked *TM*) 30 min before and during labeling with [³⁵S]methionine for 2 h. The cells were lysed in 1% SDS, samples immunoprecipitated with anti-PK monospecific IgG, and analyzed on 8% polyacrylamide gels containing 4 M urea. Lanes *U*, immunoprecipitates from uninfected cells showing endogenous monkey PK. Lanes *HN*, samples prepared as above from SV-40 recombinant virus-infected cells expressing the HN protein and immunoprecipitated with anti-HN IgG. (*Open arrows*) PK; (*solid arrows*) APK species; (*arrowhead*) glycosylated APK*; (*) nonglycosylated APK*; (<) HN species. (D) SV-40 recombinant virus-infected cells were labeled with [³⁵S]methionine for 2 h, lysed in 1% SDS, and immunoprecipitated with anti-PK IgG. Antigen-antibody protein A complexes were dissociated from the agarose beads, and not digested (lanes *CON*) or digested with Endo-F (lanes *Endo-F*). Lanes *HN*, similarly prepared cells expressing HN and immunoprecipitated with anti-HN IgG. (*Open arrow*) PK; (*solid arrow*) APK; (*arrowhead*) glycosylated APK*; (*) nonglycosylated APK*; (<) HN. In C and D only the relevant sections of the autoradiographs are shown.

analyzed by SDS-PAGE. As shown in Fig. 2 (A), PK expressed from the SV-40 vector is of the expected size (M_r ~58,000), APK was found as two closely migrating species of M_r ~65,000 and ~67,000, and APK* as a major species of M_r ~63,000 and a minor species of M_r ~65,000. HN expressed from the same SV-40 vector and immunoprecipitated with anti-HN monospecific IgG (Paterson et al., 1985) was included as a convenient size marker (M_r ~67,000).

Glycosylation of APK and APK*

To determine whether APK (or APK*) was modified by the addition of N-linked glycans, which would provide evidence that the signal/anchor domain of HN is functioning to transport the hybrid protein across the ER membrane, cells expressing APK and APK* were labeled with [³H]mannose and immunoprecipitated. Both the upper and lower APK

species were labeled with [³H]mannose (Fig. 2 B, lane *APK*). It can also be seen in Fig. 2 (B, lane *APK**) that two minor species of APK* were labeled with [³H]mannose even though APK* lacks an Asn-X-Ser/Thr sequence for the normal addition of N-linked glycosylation. Evidence is presented below that suggests that the major nonglycosylated APK* species comigrates with a very minor species of heavily glycosylated APK*.

APK and APK* synthesized in the presence of tunicamycin (TM), the inhibitor of N-linked glycosylation, have major species of identical mobility (M_r ~63,000) (Fig. 2 C, lanes *TM*) and these species comigrate with the major unglycosylated APK* band observed in the untreated control lane (Fig. 2 C). (Minor species of lower molecular mass were also found and these may represent proteolytic products.) Treatment of APK and APK* with Endo-F also yielded major species of identical mobility (M_r ~63,000) (Fig. 2

D). As expected the wild-type form of PK does not change its mobility when synthesized in the presence of TM or on digestion with Endo-F (Fig. 2 D). HN synthesized in the presence of TM and HN after digestion with Endo-F were included as a control for the assays and as a convenient size marker (Fig. 2). The sensitivity of the glycans to Endo-H digestion was also determined and after a 15-min labeling period, followed by a chase period of up to 3 h, it was found that the carbohydrate chains on APK are always sensitive to Endo-H digestion (data not shown). Thus, the N-linked carbohydrate modification of APK remains in the high mannose form and the nature of the difference in carbohydrate composition between the *M*, ~65,000 and ~67,000 forms of APK is not explained.

While the unexpected glycosylation of APK* appears to be asparagine N-linked as it is Endo-F and Endo-H sensitive and its addition is inhibited by TM, the nature and location of the oligosaccharide is not fully understood. To rule out the possibility that a mutation had arisen in APK* during subcloning or the mutagenesis procedure to form a consensus site for N-linked glycosylation (Asn-X-Ser/Thr) the entire nucleotide sequence of the cDNA insert encoding APK was obtained by the chemical method (Maxam and Gilbert, 1980). No changes in the published sequence of Lonberg and Gilbert (1983) were found, suggesting that a small percentage of APK* is glycosylated at a site other than Asn-X-Ser/Thr. This glycosylated form of APK* complicates the analysis of the intracellular transport and cell surface expression of unglycosylated APK* but, as described below, its presence does not affect the major conclusions drawn from these data.

APK and APK* Have Properties of Integral Membrane Proteins

The glycosylation of APK indicates that the HN signal/anchor domain functions as a signal for translocation of PK into the lumen of the ER. To determine if this domain acts as a stop-transfer sequence and anchors PK in membranes, microsomes were prepared from cells infected with the SV-40 recombinant viruses expressing APK, APK*, or HN, and alkaline extracted at pH 11, and separated by centrifugation through a sucrose cushion into pellet and supernatant fractions. Under these conditions integral membrane proteins fractionate in the pellet while peripheral membrane proteins and soluble proteins are found in the supernatant (Steck et al., 1973; Gilmore and Blobel, 1985; Paterson and Lamb, 1987). As shown in Fig. 3, the vast majority of all forms of APK, APK*, and the control HN sample fractionate with the membrane pellet on alkaline extraction further indicating that they are integral membrane proteins.

APK and APK* Are Expressed at the Cell Surface

To examine whether APK and APK* are expressed at the cell surface, infected cells were examined by indirect immunofluorescence using rabbit monospecific pyruvate kinase antisera and fluorescein-conjugated goat anti-rabbit IgG. Wild-type PK (Fig. 4 A) showed no detectable surface fluorescence as expected for the cytoplasmic location of the polypeptide but both APK (Fig. 4 C) and APK* (Fig. 4 E) were clearly detected on the surface of infected cells. Staining of acetone-permeabilized infected cells showed that the diffuse cytoplasmic staining pattern of wild-type PK (Fig. 4 B) could be

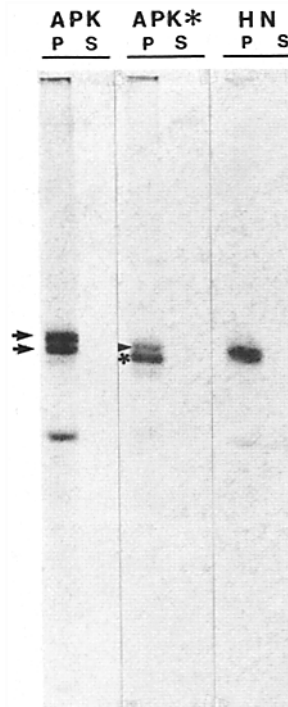


Figure 3. APK and APK* have properties of integral membrane proteins. Alkali treatment of microsomes containing APK, APK*, and HN. Microsomes isolated from SV-40 recombinant virus-infected cells labeled with [³⁵S]-methionine were treated with alkali at pH 11.0, and fractionated by centrifugation through an alkaline (0.2 M sucrose cushion). The soluble (S) and pellet (P) (membrane bound) fractions were immunoprecipitated and analyzed on 8% polyacrylamide gels containing 4 M urea. In lanes APK, two arrows indicate glycosylated species of APK; in lanes APK*, arrowhead and star indicate glycosylated and major unglycosylated species of APK* respectively; in lanes HN, < indicates HN.

readily distinguished from the staining pattern of APK (Fig. 4 D) and APK* (data not shown), which show both reticular fluorescence characteristic of the ER and intense perinuclear Golgi staining. Permeabilized uninfected cells were used as a control and they did not exhibit any detectable fluorescence (Fig. 4 F).

To provide biochemical evidence for the cell surface expression of APK and APK*, intact cells were subjected to proteolysis. CV1 cells infected with SV-40 recombinant viruses were labeled with [³⁵S]methionine for 60 min, and incubated in DME for a further 3 h to allow for transport of the proteins, and then treated with trypsin as described in Materials and Methods. To provide an internal marker, which is unaffected by trypsin digestion so that loss of cells could be controlled, cells expressing wild-type PK were mixed with those expressing either APK or APK* before the addition of trypsin. As shown in Fig. 5 A, both glycosylated APK and unglycosylated and glycosylated species of APK* are removed. Thus, all forms can be expressed at the cell surface. To obtain information concerning the amount of the APK and APK* species expressed at the cell surface and to estimate their rate of transport, CV1 cells infected with the recombinant viruses were labeled with [³⁵S]TranS-label for 15 min, incubated in DME for varying periods, and the cells subjected to proteolysis as described above. Quantitation of the autoradiograms by scanning densitometry and normalization to the wild-type PK internal control indicates that 75–85% of APK species and APK* species are accessible to trypsin digestion and that the half-time of transport to the cell surface is ~75–90 min. (Fig. 5 B). It is interesting to note that the glycosylated APK species are transported slightly more efficiently and more quickly than unglycosylated APK*. Although the kinetic analysis has not been pursued beyond 3 h of chase, the plateauing of the curve suggests that some

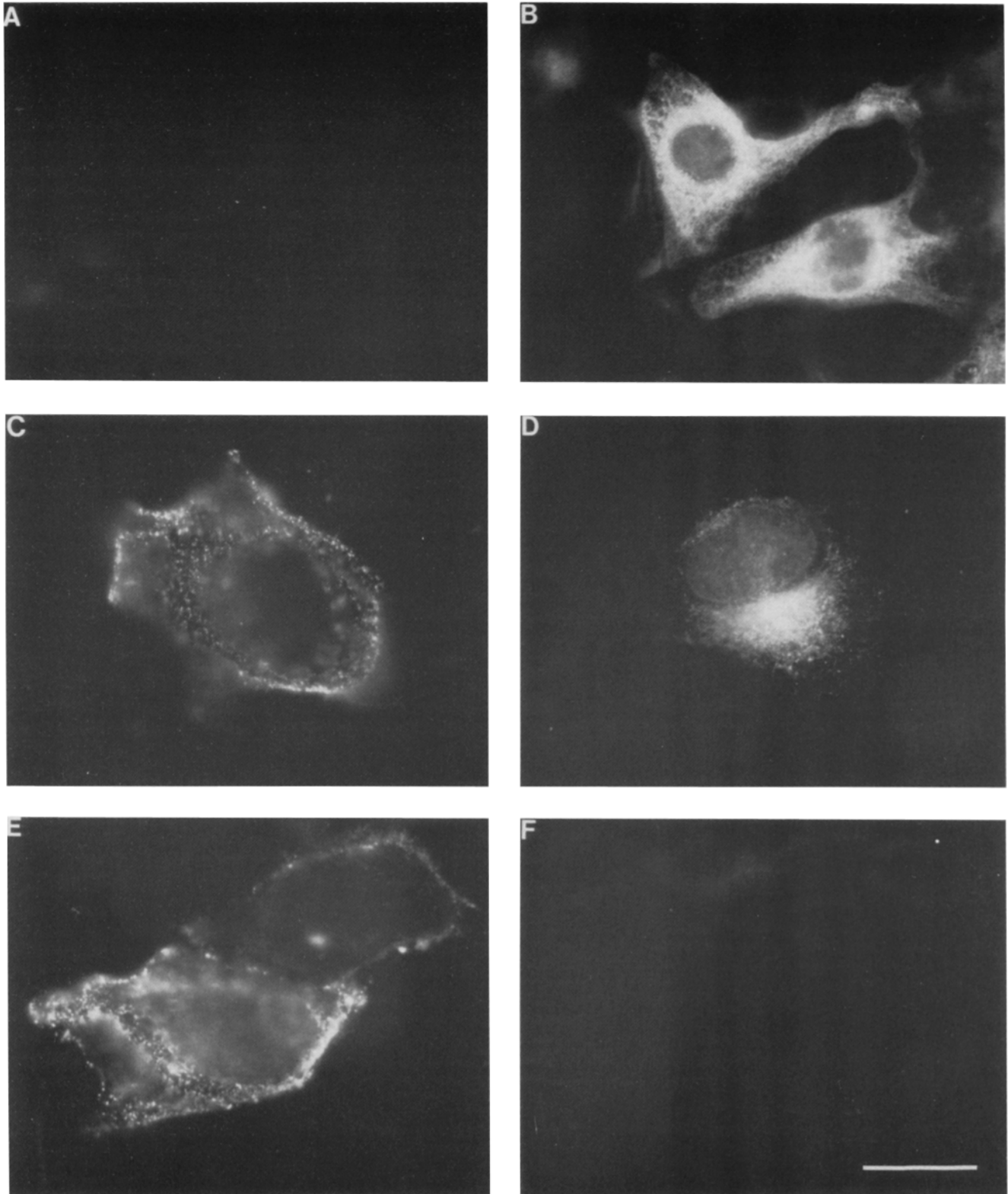


Figure 4. Indirect immunofluorescence of cells expressing the PK, APK, and APK* proteins. Cells infected with SV-40 recombinant viruses were fixed at 48 h after infection with 0.1% paraformaldehyde for 5 min. For surface fluorescence, fixed cells were incubated with monospecific anti-PK IgG followed by staining with FITC goat anti-rabbit IgG. For intracellular fluorescence, the paraformaldehyde fixed cells were permeabilized in acetone at -20°C for 2 min before binding of primary and secondary antibodies. (A, C, and E) surface staining of cells expressing PK, APK and APK*, respectively. (B, D, and F) staining of permeabilized cells expressing wild-type PK, APK, and mock-infected cells, respectively. Exposures of A were manually adjusted to be the same as C and E, and exposures of F were adjusted to be the same as B and D. In C and E, the fluorescence pattern was uniform over the cell but the microscope was adjusted such that the edges were in focus. Bar, $\sim 10\ \mu\text{m}$.

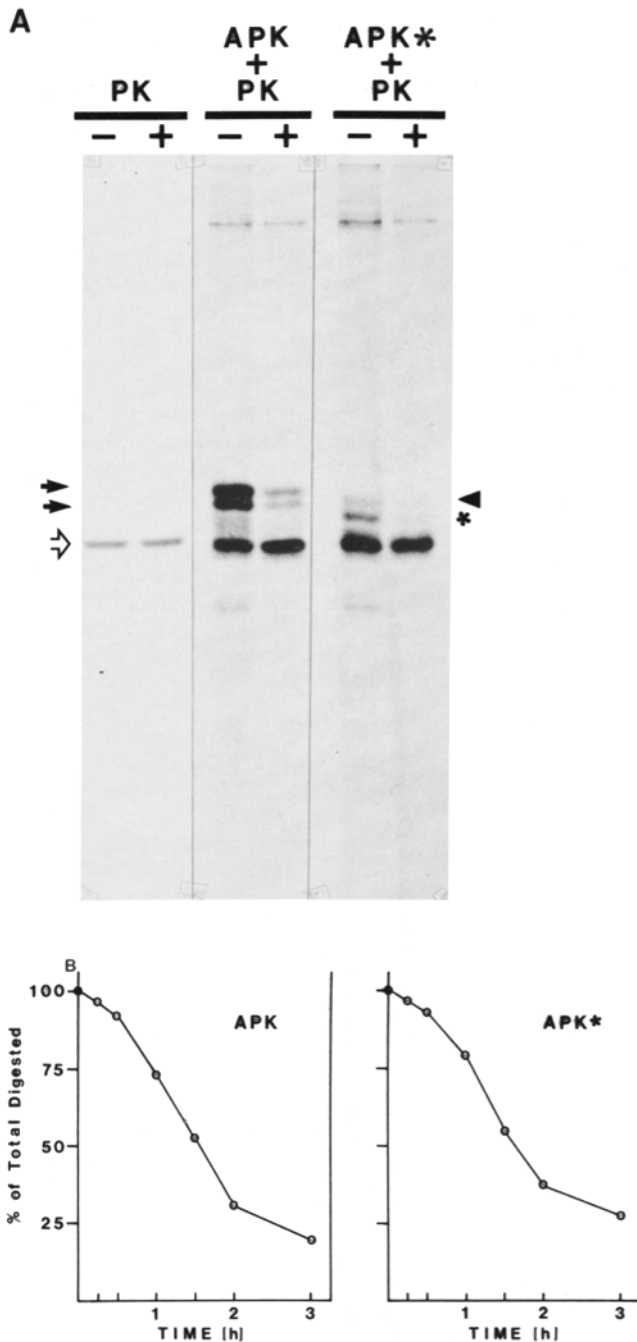


Figure 5. Trypsin treatment of the surface of cells expressing PK, APK, and APK*. (A) SV-40 recombinant virus-infected cells were labeled for 1 h with [³⁵S]methionine followed by a 3-h incubation in DME (chase). Intact cells expressing PK, APK, or APK* were either not treated (-) or treated (+) with trypsin. To both APK and APK* samples, cells expressing wild-type PK were added before the addition of trypsin to provide an internal marker to control for cell lysis. In lanes *APK*, arrows denote the position of the glycosylated APK species; in lanes *APK**, the arrowhead and star indicate the positions of the minor glycosylated and major nonglycosylated form of *APK** respectively; in lanes *PK*, *APK*, and *APK**, the open arrow indicates wild-type PK. (B) SV-40 recombinant virus-infected cells were labeled for 15 min with [³⁵S]TranS-label followed by incubation in DME for varying times. Cells were then treated with protease as described above and the autoradiograms quantitated by densitometry.

molecules of APK and APK* fail to become protease accessible at the cell surface.

Expression of Extensively Truncated APK and APK*

To gain an indication of whether the entire APK or APK* molecules attached to the cytoplasmic tail and signal/anchor domain of HN were necessary for cell surface expression, or if only a portion of APK was needed, two COOH-terminal truncations of the PK, APK, and APK* molecules were made. T₁ truncations contain 180 residues, and T₂ truncations 115 residues respectively of the original 529 residue PK molecule (see Fig. 1 and Materials and Methods). The T₁ truncations contain in addition 10 unrelated SV-40-specified residues at their COOH-terminus. SV-40 recombinant viruses were made for both the T₁ and T₂ sets of truncations in PK, APK, and APK*, and as shown in Fig. 6, the mono-specific antisera to denatured pyruvate kinase immunoprecipitates the T₁ and T₂ truncated proteins.

The N-linked glycosylation site at residue 74 has not been removed in either T₁APK or T₂APK and, therefore, it was expected that provided the HN signal/anchor domain was mediating translocation of the truncated hybrid proteins into the lumen of the ER, the expressed polypeptides would be glycosylated as was found for APK (Fig. 2). It could not be predicted whether the truncations would eliminate the small amount of unorthodox glycosylation on the mutant (APK*) that lacks a site for normal N-linked-glycosylation.

As shown in Fig. 6 A, T₁APK was found to migrate on gels as two bands (*M_r* ~30,000 and ~32,000, indicated by stars) and both these species were labeled with [³H]mannose (the mobilities are artifactually distorted because a separate gel was used for the analysis) and on Endo-F treatment T₁APK was converted to a single major band of *M_r* ~27,000 (indicated by an open arrow). T₂APK also migrated on gels as two bands (*M_r* ~22,000 and ~24,000) (Fig. 6 B) and on Endo-F treatment T₂APK was converted to a single band of *M_r* ~19,000. Thus, T₁APK and T₂APK are glycosylated much like their parent hybrid protein APK.

T₁APK* was found to migrate on gels (Fig. 6 A) as a major species of *M_r* ~27,000, that is not labeled with [³H]mannose and has the same mobility as T₁APK* treated with Endo-F. In addition two minor higher molecular mass T₁APK* species (*M_r* ~28,000 and 30,000) (indicated by a dash) were observed which could be labeled with [³H]mannose and were lost on Endo-F digestion. The *M_r* ~28,000 glycosylated form of T₁APK* comprises only a very small portion of the [³⁵S]methionine-labeled material but incorporates nearly 50% of the [³H]mannose found in APK* species. T₂APK* also migrates on gels (Fig. 6 B) as a major species of *M_r* ~19,000 and two minor species (*M_r* ~20,000 and ~21,000). These minor species of T₂APK* are lost on Endo-F digestion. Thus, these data suggest that the small amount of unexpected glycosylation that was found on APK* (Fig. 2) also occurs on 165 and 240 amino acid-containing truncated forms of APK*. This glycosylation is Endo-F and tunicamycin sensitive (Figs. 2 and 6) and is insensitive to 1 M hydroxylamine, a treatment which hydrolyzes many esterified carbohydrate linkages (Gray, 1982), suggesting that this glycosylation on <10% of the APK* molecules may be asparagine linked (data not shown). In Fig. 6 A, expression of chicken PK is used as a control marker and

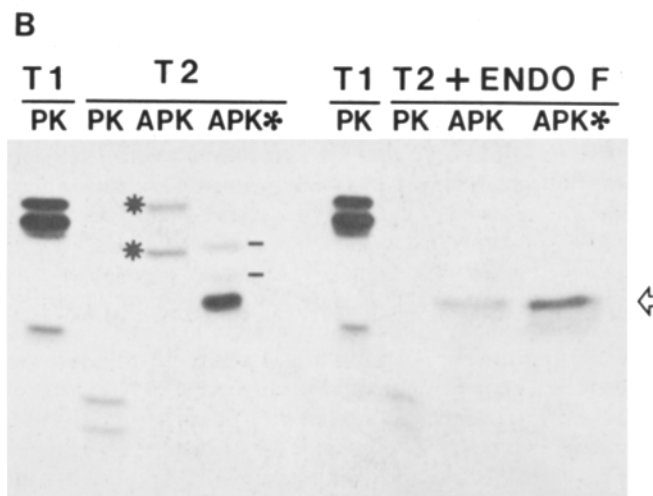
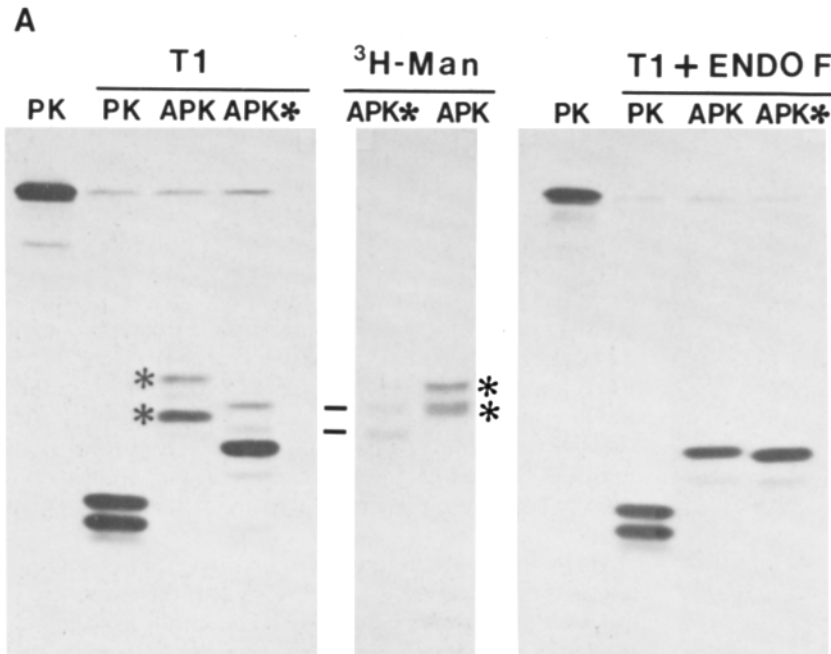


Figure 6. Expression and glycosylation of the T₁ and T₂ forms of PK, APK, and APK*. (A) Properties of the T₁, truncated forms of PK, APK, and APK*. SV-40 recombinant virus-infected cells were labeled with [³⁵S]methionine or [³H]mannose and immunoprecipitated with anti-PK monospecific IgG. Lanes T₁, [³⁵S]methionine labeled T₁ samples; lanes ³H-Man, [³H]mannose-labeled T₁ samples, and lanes T₁ + Endo-F, [³⁵S]methionine-labeled T₁ immunoprecipitates treated with Endo-F. Lanes PK, expression of chicken PK from SV-40 vector as a marker for endogenous simian PK immunoprecipitated from CV1 cells. Samples were analyzed on a 15% polyacrylamide gel. Stars indicate glycosylated forms of T₁APK, dashes indicate glycosylated forms of T₁APK* and open arrow indicates unglycosylated T₁APK* or deglycosylated T₁APK respectively. (B) Properties of the T₂ truncated forms of PK, APK, and APK*. SV-40 recombinant virus infected cells were labeled with [³⁵S]methionine and immunoprecipitated with anti-PK monospecific IgG. Lanes T₂, [³⁵S]methionine-labeled T₂ immunoprecipitates; and lanes T₂ + ENDO-F, [³⁵S]methionine-labeled T₂ immunoprecipitates, treated with Endo-F. Lanes T₁PK indicate mobility of T₁ truncated form of PK used as a size marker. Samples were analyzed on a 17.5% polyacrylamide gel containing 4 M urea. Stars indicate glycosylated forms of T₂APK, dashes indicate glycosylated forms of T₂APK* and open arrow indicates unglycosylated T₂APK or T₂APK*.

it can be seen that low levels of endogenous simian PK was also immunoprecipitated from the CV1 cells with the anti-chicken PK sera. Truncated forms of PK (T₁PK and T₂PK) were also expressed and they migrate as doublets. This probably represents the first step in proteolytic degradation, as they were found to be relatively unstable.

Truncated Forms of APK and APK* Are Expressed at the Cell Surface

To examine whether the T₁ truncations of APK and APK* are transported to the cell surface, indirect immunofluorescence was done. Bright surface staining was detected in cells expressing T₁APK and T₁APK* (Fig. 7, A and B) but no surface fluorescence was observed in cells expressing T₁APK or in uninfected cells (Fig. 7, C and D). Similar fluorescent staining was observed with cells expressing the T₂ truncations (data not shown).

To provide further evidence for the cell surface expression of the T₁ and T₂ truncated forms of APK and APK* cell surfaces were subjected to proteolysis. Cells expressing wild-type PK were mixed with cells expressing the truncated forms of APK and APK* to correct for cell lysis during the procedures. (The truncated forms of PK could not be used as a control because they are unstable in the cytoplasm.) As shown in Fig. 8 A, after a 1-h pulse label and 3-h chase period to allow transport to the cell surface, 75% of T₁APK, 62% of T₁APK*, 45% of T₂APK, and 40% of T₂APK* could be removed from the cell surface by proteolysis. To estimate their rate of transport, CV1 cells infected with the recombinant viruses were labeled with [³⁵S]TranS label for 15 min, incubated in DME for varying periods, and the cells subjected to surface proteolysis. As shown in Fig. 8 B, the recombinant molecules accessible to protease digestion all have similar half-times of transport (75–100 min). The plateauing of the curves suggests that the protease-inaccessi-

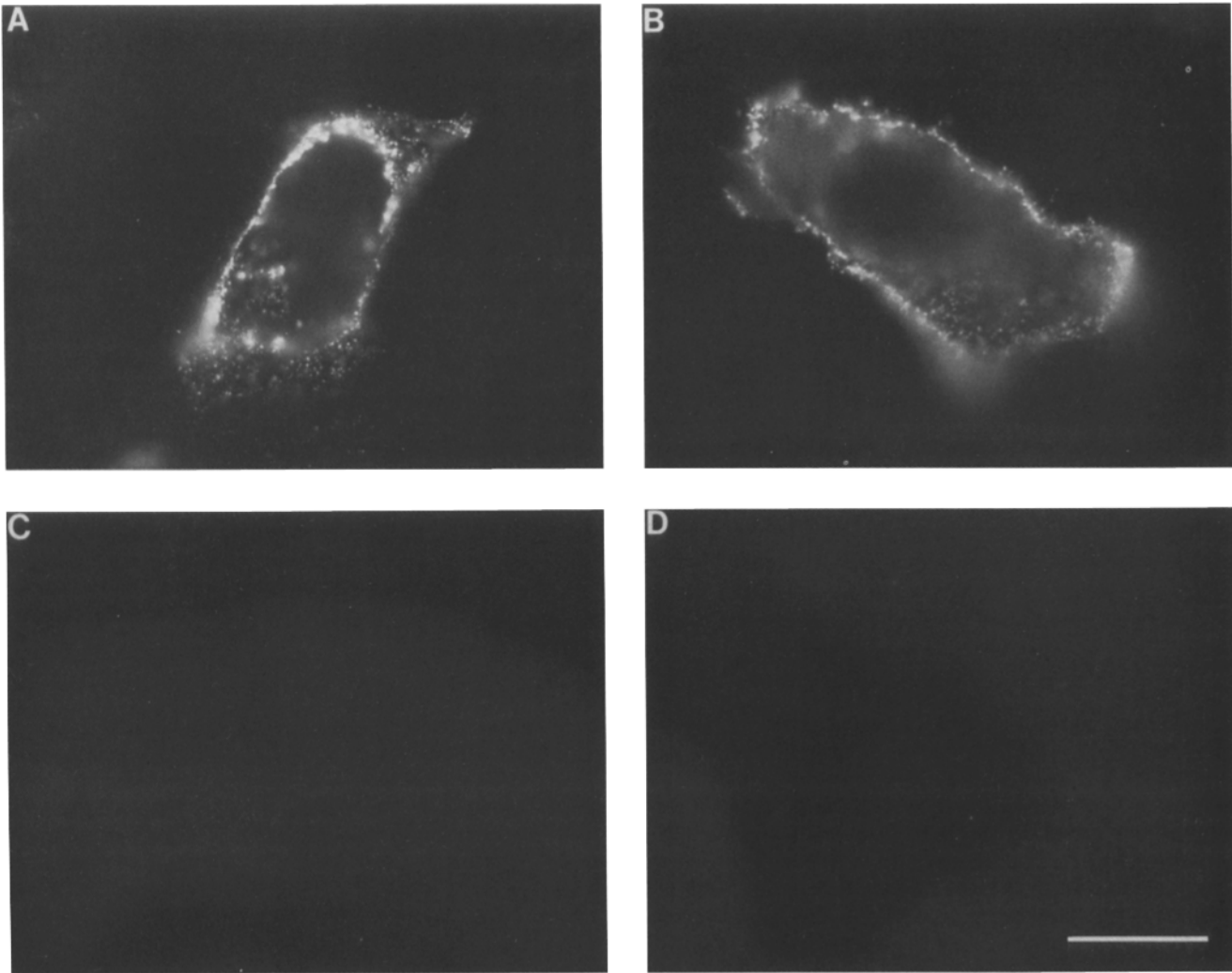


Figure 7. Indirect immunofluorescence of the surface of cells expressing the T₁ truncations of PK, APK, and APK*. Cells infected with SV-40 recombinant viruses were fixed in 0.1% paraformaldehyde and stained with anti-PK monospecific antisera and FITC-conjugated goat anti-rabbit antisera. (A) T₁ truncation of APK; (B) T₁ truncation of APK*; (C) T₁ truncation of PK; (D) uninfected cells. The exposures of C and D were manually adjusted to be the same as for A and B. Focusing of cells was as described in legend to Fig. 4. Bar, ~10 μm.

ble molecules fail to be transported to the cell surface. As noted with APK and APK*, it can be seen that glycosylated T₁APK and T₂APK molecules are transported slightly more efficiently and at a slightly faster rate than the unglycosylated species.

Discussion

The glycolytic enzyme, pyruvate kinase, has been converted from a soluble cytoplasmic protein to an integral membrane protein and expressed at the cell surface using the NH₂-terminal hydrophobic domain of the HN protein of SV5 to act as both a signal sequence for translocation across the ER membrane and as a membrane anchor. Cytoplasmic enzymes such as pyruvate kinase would not be expected to contain sorting signals for cell surface expression and it appears that the NH₂-terminal domain of HN is both necessary and sufficient to control the cell surface expression of anchored pyruvate kinase (APK): the caveat is that PK could contain

a cryptic signal for surface expression and unexpected random cryptic signal peptides have been detected in the yeast genome (Kaiser et al., 1987). However, as the T₂ truncated APK is expressed at the cell surface, any cryptic signal would have to be in the first 115 amino acid residues of PK.

It has been suggested that N-linked glycosylation may play a role in the intracellular sorting of some integral membrane proteins (Guan et al., 1985; Machamer et al., 1985). The site (Asn-Phe-Ser, residues 74-76) for N-linked glycosylation in APK is used for the addition of high mannose carbohydrate chains and when this site was changed to Asn-Phe-Ala to eliminate N-linked glycosylation (Machamer et al., 1985) in the mutant APK*, the nonglycosylated protein could still be expressed at the cell surface. However, both the rate of transport and efficiency of expression were slightly reduced when the carbohydrate was removed. There are a growing number of examples of integral membrane proteins that are transported to the cell surface regardless of glycosylation (e.g. Hickman and Kornfeld, 1978; Gibson et al.,

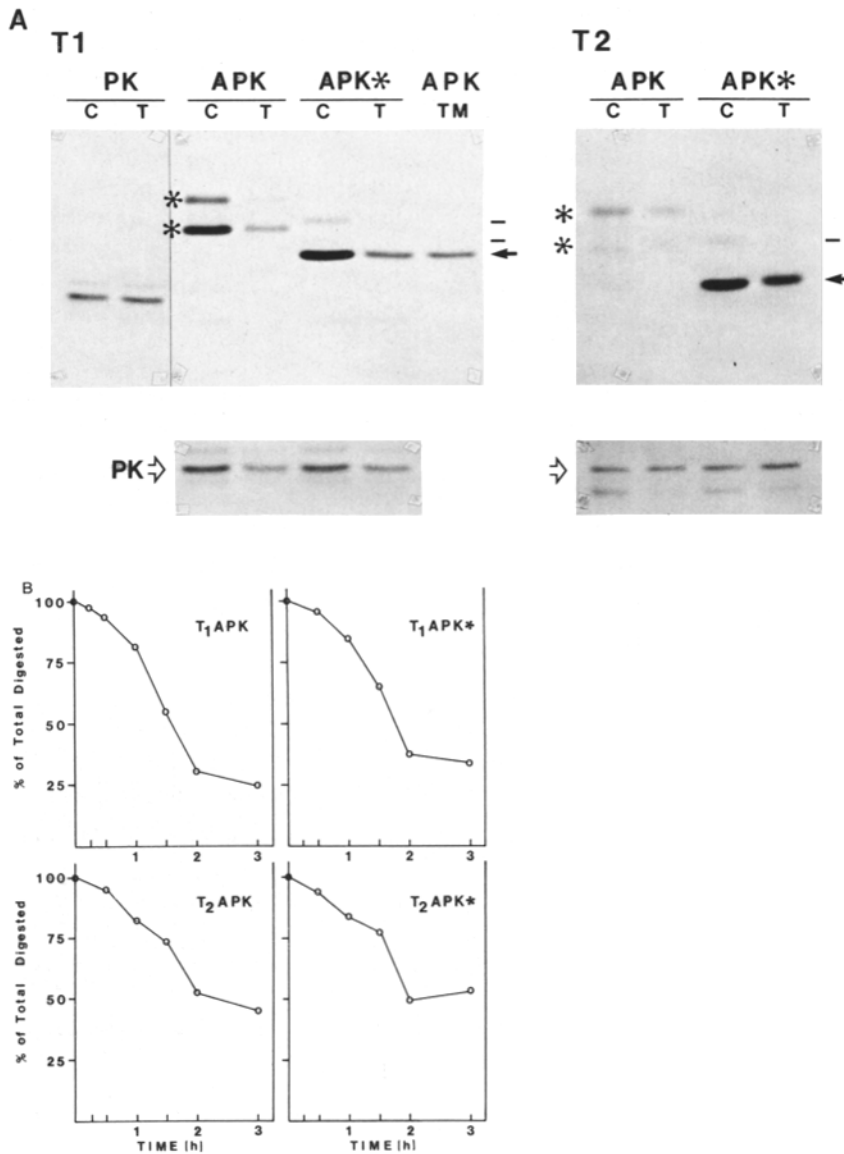


Figure 8. Trypsinization of the surface of cells expressing the T₁ and T₂ truncations of APK and APK*. (A) SV-40 recombinant virus-infected cells were labeled for 1 h with [³⁵S]methionine and incubated in DME for a further 3 h. Intact cells were either not treated (C) or treated (T) with trypsin. To both APK and APK* samples (T₁ or T₂ truncations), cells expressing wild-type PK were added before the addition of trypsin to provide an internal control for cell lysis during the procedure. Those portions of the autoradiograms showing the PK expression required a separate exposure and are shown in the lower panels with an open arrow indicating wild-type PK. Stars indicate glycosylated forms of T₁APK or T₂APK, dashes indicate glycosylated forms of T₁APK* and T₂APK* and arrows indicate unglycosylated forms of T₁APK* and T₂APK*. A marker lane of T₁APK synthesized in the presence of tunicamycin (TM) is included as a control for the mobility of the unglycosylated species of T₁APK. (B) SV-40 recombinant virus-infected cells were labeled for 15 min with [³⁵S]Trans-label followed by incubation in DME for varying times. Cells were then treated with protease as described above and the autoradiograms quantitated by densitometry. The integrated peak values of all T₁ or T₂APK species were combined but only the unglycosylated T₁ or T₂APK* species were used.

1978; Hannik and Donoghue, 1986; Santos-Aguado et al., 1987). For those proteins for which carbohydrate seems necessary for intracellular transport, it is possible that rather than acting as a positive signal for transport, the addition of glycans may aid in the solubility of the proteins or aid in the formation of the native conformation of the protein, which in turn regulates cell surface localization (Machamer et al., 1985).

The data concerning the cell surface localization of the nonglycosylated form of APK* are complicated by the finding that a small proportion (<10%) of APK* molecules are modified by the apparent addition of N-linked oligosaccharides. However, in the truncated forms of APK* (T₁ and T₂) the minor glycosylated species can be readily separated on gels from the major nonglycosylated species. Protease digestion of the proteins expressed at the cell surface demonstrates that the nonglycosylated form of T₁APK* and T₂APK* can be found at the cell surface. Thus, glycosylation is not absolutely required for the intracellular transport of these forms

of APK*. The precise nature and location of this unexpected oligosaccharide addition are unknown.

Studies on the intracellular oligomerization and time course of translocation of influenza virus hemagglutinin and VSV G protein have indicated that these glycoproteins form multimeric structures before transport from the ER (or perhaps the Golgi apparatus) to the cell surface (Gething et al., 1986; Copeland et al., 1986; Kreis and Lodish, 1986). It has been inferred from these data and the observation that many hybrid proteins constructed to probe the intracellular sorting mechanisms failed to be transported (reviewed by Garoff, 1985), that the correct folding of integral membrane proteins into their mature conformation may be a prerequisite for transport of proteins to the cell surface (Gething et al., 1986). It is not known if APK would fold into the native tertiary structure of PK because of its attachment to membranes and its location in an environment other than that of the cytoplasm. Unfortunately assays for biologically active SV-40-expressed PK and APK were negative, monoclonal anti-

bodies to specific domains are presently unavailable, and biophysical assays would be impractical. However, it is doubtful that APK can be native because using the known x-ray crystallographic structure of PK (Muirhead et al., 1986) it can be predicted that the N-linked carbohydrate would be attached very close to the active site of PK and it could not be sterically accommodated in a native molecule. Although it has not been shown directly, it seems reasonable to suggest that the extensively truncated forms of APK (T₁APK and T₂APK) are not in a native form and they may assume more than one tertiary structure. None the less, a portion of the molecules can be expressed at the cell surface.

Several models for the intracellular sorting of proteins to the cell surface have been proposed (e.g., reviewed in Pfeffer and Rothman, 1987). Transport of integral membrane proteins to the cell surface may be mediated through receptors that recognize positive-acting signals. These receptors would be responsible for concentrating the protein into transport vesicles before export. Evidence in support of this mechanism has been derived from the differing rates of transport of both integral membrane proteins and secretory proteins (e.g., Lodish et al., 1983, 1987; Blumberg et al., 1985). In an alternative mechanism, it is suggested that there are no signals or receptors for transport out of the ER to the cell surface but that "bulk flow" is the major process for translocation with the sorting mechanism being mediated by the selective retention of proteins in the ER or Golgi apparatus (Rothman, 1987; Pfeffer and Rothman, 1987; Wieland et al., 1987). This proposal is based on the rate of transport of a synthetic glycopeptide which is thought unlikely to contain transport signals, and is rapidly secreted from the cell (Wieland et al., 1987). Thus, positive-acting transport signals may not be needed and intracellular sorting may occur by retention of proteins in the ER and Golgi apparatus (Rothman, 1987; Pfeffer and Rothman, 1987; Wieland et al., 1987). This view has gained support with the finding of sequences within resident ER proteins which act as retention signals (Poruchynsky et al., 1985; Munro and Pelham, 1987; Paabo et al., 1987). The finding of very small ectodomains in small integral membrane proteins that are expressed at the cell surface (e.g., influenza A virus M₂ protein (23 residues), influenza B virus NB protein (17 residues), and SV5 SH protein (~5 residues) (Hiebert et al., 1985b; Lamb et al., 1985; Zebedee et al., 1985; Williams and Lamb, 1986; Hiebert et al., 1988), also makes it less likely that complex positive-acting signals are required in the ectodomain for cell surface localization. However, the above proteins contain more extensive cytoplasmic domains which could contain transport signals, although in many proteins studied complete removal of the cytoplasmic tail does not block intracellular transport (e.g., Davis et al., 1983; Zuniga and Hood, 1986; Doyle et al., 1986; Puddington et al., 1986). Correct folding and oligomerization may be a prerequisite for the transport of some proteins (e.g., IgG heavy chain, HA, VSV G) but this may be because incorrectly folded forms of these proteins expose a retention signal that interacts in the ER with resident proteins such as BiP (the IgG heavy chain binding protein, also known as GRP78; Bole et al., 1986; Kreis and Lodish, 1986; Munro and Pelham, 1986; Gething et al., 1986) and this signal is buried on correct folding. The interpretation of our data concerning the expression of full-

length and truncated forms of pyruvate kinase at the cell surface that we favor, given the caveat concerning cryptic signals in the 115 NH₂-terminal residues of PK, is that positive-acting signals are not required in the ectodomain for cell surface localization. Thus, if this is the case then transport signals either reside within the 48 amino acids of the NH₂-terminal domain of HN or transport to the cell surface of membrane-anchored pyruvate kinase is by a default pathway.

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