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) (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 the 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 carbohydrate 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 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, ~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 XhoI digestion and the single BglI site in the HN CDNA was changed to EcoRI using BglI digestion, treatment with T4 DNA polymerase, and ligation of EcoRI linkers. PK cDNA clone pHLL2PK10 (Lonsberg 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 BglI site at the 3' terminus of the PK cDNA was changed to an XhoI site by BglII digestion, treatment with T4 DNA polymerase, and ligation of XhoI linkers. The APK hybrid gene was constructed by ligation of the small XhoI to EcoRI HN DNA fragment encoding the first 48 NH2-terminal amino acids of HN to the large EcoRI to XhoI 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 residues 1-48 were joined to PK residues 17 to 529, by sequencing plasmid DNA using the dye oxynucleotide 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 XhoI cloning site instead of BamHI. As a control, the chicken PK gene was expressed in the pSV63 vector. The PK cDNA insert from pHLL2PK10 (Kalderon et al., 1984) was isolated by Hind III and BglII digestion, treatment with T4 DNA polymerase, addition of XhoI 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-Pho-Ser in APK (residues 74-36) was altered to Asn-Pho-Ala by site specific mutagenesis. APK DNA was subcloned into the replicative form of bacteriophage M13mp19 which is a derivative of M13mp9 containing an XhoI site in place of the BamHI site in the polylinker 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 dye oxynucleotide chain-terminating sequencing of the M13mp9XAPK* single-strand DNA (Sanger et al., 1977). The APK* mutant DNA insert was isolated from replicate form of M13mp9XAPK* 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 XhoI to SalI (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).

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 XhoI 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-
Metabolic Labeling of Polypeptides, Immunoprecipitation, and SDS-PAGE

SV-40 recombinant-virus infected CVI cells were labeled with \[^{35}S\]methionine or \[^{35}S\]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 uCi \[^{3}H\]mannose in glucose-free MEM per 1-cm-diam well of CVI 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 Choppin, 1976).

Cell Surface Trypsinization

Confluent monolayers of CVI cells (10-cm-diam plates) were infected with SV-40 recombinant viruses and at 44 h after infection the cells were labeled with \[^{35}S\]methionine for 15 min, the label removed, and the cells incubated for varying periods up to 3 h (chase) in DMEM. Cells were scraped from the plates with a silicon rubber policeman, resuspended in PBS, and any transport signals present in pyruvate kinase are 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 A 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 NH\_2 terminus. Previously, residues encoding the SV-40 T antigen nuclear localization signals have been added to the NH\_2 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 NH\_2 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.

Indirect Immunofluorescence

Coverslips of SV-40 recombinant virus-infected CVI 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. For endoglycosidase H (Endo-H) analysis, SV-40 recombinant virus-infected CVI cells were pulse labeled with \[^{35}S\]methionine and incubated in DMEM (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

CVI cells infected with SV-40 recombinant viruses were treated with 3 μg/ml tunicamycin (Calbiochem-Behring Corp., La Jolla, CA) in DMEM 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 NH\_2-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 A 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 NH\_2 terminus. Previously, residues encoding the SV-40 T antigen nuclear localization signals have been added to the NH\_2 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 NH\_2 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 NH\_2-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 CVI cells together with the SV-40 early region mutant d11055 (Pipas et al., 1983), and viral stocks were made. The proteins were immunoprecipitated from \[^{35}S\]methionine-labeled infected CVI cells with anti-chicken pyruvate kinase monospecific IgG and

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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, ~58,000), APK was found as two closely migrating species of M, ~65,000 and ~67,000, and APK* as a major species of M, ~63,000 and a minor species of M, ~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, ~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, ~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, ~63,000) (Fig. 2
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 $M_r \sim 65,000$ and $\sim 67,000$ 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 Gilber (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 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. CVI 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, CVI 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 halftime of transport to the cell surface is $\sim\text{75–90 min}$. (Fig. 5 B). It is interesting to note that the glycosylated APK species are transported slightly more efficiently and more quickly than unglycosylated APK*.
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, ~10 μm.
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.

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 monospecific 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 (Mr ~30,000 and ~32,000, indicated by stars) and both these species were labeled with [3H]mannose (the mobilities are artificially distorted because a separate gel was used for the analysis) and on Endo-F treatment T1APK was converted to a single major band of Mr ~27,000 (indicated by an open arrow). T2APK also migrated on gels as two bands (Mr ~22,000 and ~24,000) (Fig. 6 B) and on Endo-F treatment T2APK was converted to a single band of Mr ~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 Mr ~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 (Mr ~28,000 and 30,000) (indicated by a dash) were observed which could be labeled with [3H]mannose and were lost on Endo-F treatment. The Mr ~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 Mr ~19,000 and two minor species (Mr ~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...
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 DMEM 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.

In soluble 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 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 \( T_1 \) and \( T_2 \) 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 (\( T_1 \) or \( T_2 \) 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 TIAPK or T2APK, dashes indicate glycosylated forms of \( T_1 \)APK and \( T_2 \)APK, and arrows indicate unglycosylated forms of \( T_1 \)APK* and \( T_2 \)APK*. A marker lane of \( T_1 \)APK synthesized in presence of tunicamycin (TM) is included as a control for the mobility of the unglycosylated species of \( T_1 \)APK. (B) SV-40 recombinant virus–infected cells were labeled for 15 min with \([35S]\)Trans-S-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 \( T_1 \) or \( T_2 \)APK species were combined but only the unglycosylated \( T_1 \) or \( T_2 \)APK* species were used.

1978; Hanks 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* (\( T_1 \) and \( T_2 \)) 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 \( T_1 \)APK* and \( T_2 \)APK* 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 with 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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