A Novel NH$_2$-terminal, Nonhydrophobic Motif Targets a Male Germ Cell-specific Hexokinase to the Endoplasmic Reticulum and Plasma Membrane*

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Although three germ cell-specific transcripts of type 1 hexokinase exist in murine male germ cells, only one form, HK1-sc, is found at the protein level. This single isoform localizes to three distinct structures in mouse spermatozoa: the membranes of the head, the mitochondria in the midpiece, and the fibrous sheath in the flagellum (Travis, A. J., Foster, J. A., Rosenbaum, N. A., Visconti, P. E., Gerton, G. L., Kopf, G. S., and Moss, S. B. (1998) Mol. Biol. Cell 9, 263–276). The mechanism by which one protein is targeted to multiple sites within this highly polarized cell poses important questions of protein targeting. Because the study of protein targeting in germ cells is hampered by the lack of established cell lines in culture, constructs containing different domains of the germ cell-specific hexokinase transcripts were linked to a green fluorescent protein and transfected into hexokinase-deficient M+R42 cells. Constructs containing a nonhydrophobic, germ cell-specific domain, present at the amino terminus of the HK1-SC protein, were targeted to the endoplasmic reticulum and the plasma membrane. Mutational analysis of this domain demonstrated that a complex motif, PKIRPRPLTE (with essential residues italicized), represented a novel endoplasmic reticulum-targeting motif. Constructs based on another germ cell-specific hexokinase transcript, HK1-sa, demonstrated the specific proteolytic removal of an amino-terminal domain, resulting in a protein product identical to HK1-SC. Such processing might constitute a regulatory mechanism governing the spatial and/or temporal expression of the protein.

The targeting of proteins to specific organelles or biochemical compartments within a cell is critical for normal cellular function. The biological significance of appropriate protein targeting is best demonstrated in cells that are highly polar in organization. For example, epithelial cells of the renal tubules and intestinal lumen could not provide directional transport without their sodium-potassium ATPases organized strictly on their basolateral surfaces. Among the various cell types that have been studied, mature spermatozoa represent one of the most highly differentiated and polarized cells. The spermatozoon can be divided into three main compartments: the head, the midpiece, and the principal piece of the flagellum. Within these different compartments are many unique organelles such as the membrane-delimited acrosome in the head, as well as the fibrous sheath and outer dense fibers, cytoskeletal elements that surround the axoneme in the flagellum. Furthermore, organelles common to both germ cells and somatic cells possess unusual adaptations in the male gamete. In this regard, sperm mitochondria differ from their somatic counterparts in that they are restricted to a specific region of the cell (the midpiece of the flagellum) and possess additional germ cell-specific isoenzymes (e.g. lactate dehydrogenase-X) (1, 2). By regionalizing the distribution of specific organelles and proteins, spermatozoa have achieved a functional compartmentalization of the machinery necessary for such diverse functions as cellular motility, binding and penetrating the extracellular matrix of the egg, and binding and fusing with the plasma membrane of the egg. How these components are targeted and assembled during spermatogenesis to form the polarized spermatozoon is largely unknown.

One enzyme critical to the compartmentalized metabolic pathways of both spermatozoa and somatic tissues is type 1 hexokinase (HK1). This enzyme is best known for catalyzing the phosphorylation of glucose in the first step of glycolysis. Targeting of the somatic isoforms of HK1 is based largely upon the presence of different amino-terminal domains. The classical somatic cell HK1 can associate with the outer mitochondrial membrane through a 15-amino acid amino-terminal hydrophobic domain (3, 4). This “mitochondrial membrane-binding domain” has been shown to be sufficient to target a green fluorescent protein (GFP) construct to the mitochondria in M+R42 cells, a HK-deficient cell line (5). It is believed that the hydrophobic nature of this domain allows insertion of this region of the protein into the outer mitochondrial membrane (6).

Two cell types, reticulocytes and male germ cells, contain mRNAs encoding variants of HK1 that do not possess this mitochondrial membrane-binding domain. In human reticulocytes, one of these HK variants contains an alternative amino terminus that replaces the first 21 residues with 20 alternative

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1 The abbreviations used are: HK1, type 1 hexokinase; AKAP, protein kinase A-anchoring protein; GFP, green fluorescent protein; GCS, germ cell-specific ER, endoplasmic reticulum; SA, a unique amino-terminal domain encoded only by the HK1-sa transcript; SB, a unique internal domain encoded only by the HK1-sb transcript; PCR, polymerase chain reaction.

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Amino acids (7). Interestingly, this reticulocyte-specific HK isozyme does not target to mitochondria but rather is found exclusively in the cytosol.

It has been known for some time that male germ cells possess a variant of HK1 protein (8, 9). In both mice and humans, three cDNAs have been identified that are predicted to encode for male germ cell-specific isoforms of HK1 that do not contain either the mitochondrial membrane-binding domain or the reticulocyte-specific sequence (10, 11). Rather, the murine germ cell-specific HK mRNAs all encode an alternative 24-amino acid domain (10), called GCS for its germ cell-specific expression. This GCS domain is not hydrophobic in nature, in contrast to the somatic mitochondrial membrane-binding domain.

Although three germ cell-specific HK1 transcripts, HK1-sa, HK1-sb, and HK1-sc, have been identified in the mouse (10), only one, HK1-SC,2 has been demonstrated to be expressed at the protein level (12). Our interest in the targeting of proteins within male germ cells began with our demonstration that this isoform is found associated with the membranes of the head of within male germ cells. This processing might offer an explanation for the presence of the desired insert. DNA from plasmids containing inserts were sequenced. Transformed cells containing appropriate inserts were grown up, and plasmids were purified using the Perfect Prep Plasmid Purification Kit according to the manufacturer's instructions (Eppendorf). The PCR products were ligated into the plasmid with T4 DNA ligase, and the PCR products were ligated into the plasmid with T4 DNA ligase.

Studies of protein targeting in germ cells have been hampered by the lack of established germ cell lines in culture. Therefore, to initiate a study of the mechanism by which HK may be targeted to multiple subcellular locations in sperm, we have chosen a heterologous cell expression system previously found to be useful in studies of the targeting of somatic forms of mammalian HK (5). A series of HK-GFP fusion constructs including different segments of the murine germ cell-specific isoforms, or mutants thereof, were expressed in the HK-deficient M+R42 cell line (15). Subcellular distribution was monitored using confocal fluorescence microscopy. The nonhydrophobic GCS domain was found to be necessary and sufficient to target fusion proteins to the ER and plasma membrane in this expression system. Mutational analysis defined a specific and conserved targeting motif located within the carboxyl-terminal 10 amino acids of the GCS domain. Individual point mutations and several combinations of mutations within this region did not abolish ER targeting but did disrupt normal protein processing through the ER to the plasma membrane. Only when six specific residues were mutated in combination was targeting to the ER abolished. Constructs based on another germ cell-specific HK1 transcript, HK1-sa, revealed the specific prolyleptic removal of the unique amino-terminal domain of this isoform. The cleavage of this domain resulted in a protein identical to HK1-SC, the isoform of HK1 previously demonstrated to be expressed in sperm (12). This processing might offer an explanation as to why HK1-sa transcript was not found at the protein level in a previous study (12) and may represent a novel regulatory mechanism governing spatial and temporal expression of this protein during spermatogenesis.

EXPERIMENTAL PROCEDURES

Vectors, Cell Lines, and Fluorescent Markers—The pEGFP-N1 vector (CLONTECH, Palo Alto, CA) was utilized for the production of HK fusion proteins with a carboxyl-terminal GFP tag. This vector permitted the expression of the fusion proteins under the control of the cytomegalovirus promoter. Plasmids were transfected into M+R42 cells, a HK-deficient Chinese hamster ovary cell line (15). The M+R42 cell line was generously provided by Drs. Michael Morgan and Pelin Faik (Guy's Hospital Medical School, London, UK). Rhodamine 123 and DioC6(3) were purchased from Molecular Probes, Inc. (Eugene, OR).

Generation of Plasmids—The cDNA clones encoding the different germ cell-specific HK1 isoforms were generously provided by E. M. Eddy (National Institutes of Environmental Health Sciences, Research Triangle Park, NC) (10). Oligonucleotide primers for PCR were based upon the published sequences from Mori et al. (10), and are listed in Table I. All constructs not containing mutations were generated by the same general method that is described below. A schematic representation of the constructs generated for use in this study is shown in Fig. 1. To aid in the interpretation of the data, constructs have been named according to the domains that they possess.

To briefly, constructs including the SA domain, or the truncated SA domain (tSA), were first amplified from mixed germ cell total RNA by reverse transcription PCR, using SuperScript II according to the manufacturer's instructions (Life Technologies, Inc.). For other constructs, the appropriate DNA regions were amplified with oligonucleotide primers using either the original cDNA clones or plasmids constructed previously as the template (12). Either Taq or Taq DNA polymerase was used depending upon the length of the expected product, according to the manufacturer's instructions (Stratagene, La Jolla, CA). All PCR products were visualized on 1% agarose gels with ethidium bromide and then isolated using the Wizard PCR Peps Purification Kit according to the manufacturer's instructions (Promega, Madison, WI). The PCR products and the pEGFP-N1 vector were digested with HindIII and EcoRI (Promega, Madison, WI, and Life Technologies, Inc., respectively). The plasmid was dephosphorylated with calf intestinal alkaline phosphatase, and the PCR products were ligated into the plasmid with T4 DNA ligase (Life Technologies, Inc.). Plasmids then were transformed into HB101 competent cells (Promega), purified, and analyzed to check for the presence of the desired insert. DNA from plasmids containing inserts were sequenced on each end and over any internal domains of interest to ensure that the insert was correct and in-frame with the carboxyl-terminal GFP. An Applied Biosystems (Foster City, CA) model 373A automated sequencer, with the BigDye Terminator Cycle Sequencing kit with AmpliTaq DNA polymerase FS, was utilized for sequencing. Transformed cells containing appropriate inserts were grown up, and plasmids were purified using the Perfect Prep Plasmid DNA kit (5 Prime → 3 Prime, Inc., Boulder, CO), prior to transfection into the M+R42 cells.

| Construct | Sense oligonucleotide primer<sup>a,b</sup> | Anti-sense oligonucleotide primer<sup>c</sup> |
|-----------|---------------------------------|-------------------------------------|
| tH        | 275–296<sup>d</sup>            | 1616–1640                           |
| GCS/H     | 78–102                          | 1616–1640                           |
| GCS       | 78–102                          | 272–296                             |
| GCS/H     | 78–102                          | 2572–2591                           |
| tSA/GCS/tH| 47–67                           | 1616–1640                           |
| SA/GCS/H  | 2–25                            | 1616–1640                           |
| tSA/GCS   | 47–67                           | 272–296                             |
| SA/GCS    | 47–67                           | 172–193                             |
| tSA       | 2–25                            | 172–193                             |
| SA        | 2–25                            | 172–193                             |
| tH/SB     | 275–296                         | 1616–1640                           |
| GCS/H/SB  | 173–190                         | 1616–1640                           |

<sup>a</sup> Nucleotides are numbered according to the sequence for HK1-sa in Mori et al. (10).
<sup>b</sup> Sense oligonucleotides contained a HindIII site at their 5′ end.
<sup>c</sup> Anti-sense oligonucleotides contained an EcoRI site at their 3′ end.
<sup>d</sup> A G at position 287 was converted to a C to remove an out-of-frame ATG.

2 Lowercase letters will be used to denote nucleic acids/transcripts (e.g. HK1-sc), and uppercase letters will be used to denote proteins (e.g. HK1-SC).
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Generation of Mutant Constructs—Point mutations in the GCS domain were created using the Quik-Change Mutagenesis kit (Stratagene, La Jolla, CA). Amino acids were altered with the goals of changing relative charge or hydrophobicity, while keeping size relatively constant. Oligonucleotide primers (Table II) were constructed to contain the desired amino acid changes that resulted in the desired expression of alternative amino acid residues. Mutant constructs were generated according to the instructions of the kit, with the exceptions that the annealing temperature was varied from 55 to 59 °C and the number of cycles was varied from 10–18, depending upon the construct. Briefly, sense and antisense primers covering the same region were generated and used for PCR off the circular plasmid. After PCR, the DNA was treated with the enzymes to digest the methylated parental plasmid. The mutated plasmid was then transformed into competent cells, isolated, and sequenced as above. Mutant constructs containing amino-terminal deletions were generated with oligonucleotide primers containing an in-frame ATG 5′ to the region where translation was to begin. The protocol for generating the construct was the same as described above for nonmutant constructs.

Cell Culture and Transfection—For observation by confocal microscopy, M+R42 cells were cultured in chambered coverglasses (Lab-Tek catalog number 178655, Nalge Nunc International, Naperville, IL) and transfected using previously described procedures (5). LipofectAMINE (Life Technologies, Inc.) was used as the transfection reagent. For preparation of cell extracts used in the immunoblotting experiments, M+R42 cells were grown in 100-mm plates. Transfection was done using LipofectAMINE PLUS to obtain higher transfection efficiencies, following the protocol supplied by the manufacturer. Extracts were prepared approximately 30 h after transfection. The cells were rinsed three times with phosphate-buffered saline, followed by the addition of 0.6 ml of hot (boiling water bath) sample buffer (16). The cell lysates were transferred to microfuge tubes, sonicated for 6 s, and centrifuged for 5 min. The resultant supernatants were used for immunoblotting.

Electrophoresis and Immunoblotting—SDS-polyacrylamide gel electrophoresis on 6–20% gradient gels and electrophoresis was performed as described previously (5). Blots were blocked with 1% gelatin-5% nonfat dry milk in Tris-buffered saline and developed by standard methods, with detection using the SuperSignal West Pico Reagent (Pierce).

Alternatively, proteins from cell lysates were separated and visualized using 10% gels (12). Briefly, protein extracts from the transfected M+R42 cells were electrophoresed using 10% gels under reducing conditions (16). Proteins were transferred from gels to Immobilon-P membranes (Millipore, Bedford, MA), which were then blocked in a Tris-buffered saline solution containing 1% Tween 20 (TTBS), and 5% cold water teleostean gelatin (Sigma). A variety of polyclonal antisera were used to detect the fusion protein products on immunoblots. Anti-GFP was purchased from CLONTECH and used at a dilution of 1:10,000. An antisera that detects both somatic and germ cell HK1 (anti-HK1) was used at a dilution of 1:1,000 (13). Antiserum against either the unique amino-terminal region of HK1-SA (anti-SA) or the germ cell-specific region shared by HK1-SA and HK1-SB, and HK1-SC (anti-GCS) were used at dilutions of 1:1,000 and 1:10,000, respectively (12). Blots were probed with the appropriate primary antibody diluted in TTBS for 1 h, washed in TTBS, and then probed with an anti-rabbit, peroxidase-conjugated secondary antibody for 5 min. Blots were washed in TTBS for at least 2 h prior to visualization of target proteins by chemiluminescence (ECL, Amersham Pharmacia Biotech) and autoradiography.

RESULTS

The Germ Cell-specific Domain of HK1-SC Targets Fusion Proteins to the ER—To examine the protein targeting of the germ cell-specific HK1 isoforms, we utilized an HK1-deficient somatic cell line, M+R42 cells, because no germ cell lines currently exist in culture that are permissive for spermatogenic differentiation. Confirming previous results (5), sham-transfected cells (transfected with vector containing no insert) showed no fluorescence when viewed under conditions used to image GFP fluorescence in cells expressing GFP-constructs (results not shown). When stained with the mitochondrial marker, rhodamine 123, mitochondria were clearly visible in a tubular-punctate pattern in these cells (Fig. 2A). Staining of cells with DioC6(3), which primarily stains the ER, revealed a reticular pattern in the perinuclear region (Fig. 2B). DioC6(3) also labeled some peri-nuclear mitochondria. However, the differences in the pattern of staining between the organelles and the comparison of this labeling to the rhodamine 123 pattern allowed the two organelles to be easily distinguished. When cells were transfected with a construct containing GFP alone, a diffuse fluorescence was observed throughout the cytoplasm and the nucleus that did not correlate with either ER or mitochondrial localization (Fig. 2C). In addition to these controls, it was necessary to ensure that constructs within the HK1-SC domain shared between the germ cell-specific and somatic isoforms did not play a role in protein targeting. It has been suggested that other regions within the amino-terminal half of HK1 might interact with the mitochondrial membrane-binding domain and confer additional targeting information (5, 17). As a control for the possibility that these domains might confer specific location.
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Oligonucleotide primers and specific nucleotide substitutions used in the generation of mutant HK-GFP fusion proteins

| Mutant construct<sup>a</sup> | Nucleotide substitutions<sup>b</sup> | Oligonucleotide primers<sup>b</sup> |
|-----------------------------|-------------------------------------|-----------------------------------|
| mGCS(K16N)V/H               | 241: A/C                            | 225–260                           |
| mGCS(R18Q)V/H               | 246: G/A                            | 225–260                           |
| mGCS(K16N,R18Q)V/H          | 241: A/C; 246: G/A                  | 225–260                           |
| mGCS(P15A)/V/H              | 236: C/G                            | 224–268                           |
| mGCS(P19A,P20A)/V/H         | 245: C/G; 251: C/G                  | 224–268                           |
| mGCS(1-21P,T22A,E23Q)/V/H   | 255: T/C; 257: A/G; 260: G/C        | 245–272                           |
| mGCS(del1–14)/V/H           | Not applicable                      |                                   |
| mGCS(P15A,K16N,R18Q,L21P,T22A,E23Q)/V/H | 236: C/G; 241: A/C; 246: G/A; 255: T/C; 257: A/G; 260: G/C | 225–260 |

<sup>a</sup> Amino acid residues are numbered from the first M in the GCS domain, from Mori et al. (10).

<sup>b</sup> Nucleotides are numbered according to the sequence for HK1-sa in Mori et al. (10).

<sup>c</sup> A 5’ ATG was added to the sense oligonucleotide primer to begin translation at residue 15.

<sup>d</sup> Sense oligonucleotides contained a HindIII site at their 5’ end.

<sup>e</sup> Anti-sense oligonucleotides contained an EcoRI site at their 5’ end.

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When imaging fields of cells transfected with the above constructs, there were several cells that showed a diffuse pattern of fluorescence typical for GFP alone. This phenomenon occurred at a higher frequency in cells transfected with the GCS/H and GCS constructs than in cells transfected with GFP/tH. One possible explanation for this cytoplasmic localization was the proteolytic removal of the GCS domain, leaving either HK-GFP or GFP alone. Indeed, when extracts of these transfected cells were examined by immunoblotting, it was clear that some proteolytic processing of the GCS region had occurred and that removal of the GCS region occurred at a higher frequency in the GCS and GCS/H constructs than in the GCS/tH construct (Fig. 4 shows processing of the GCS construct as an example of this phenomenon). It has been established previously that the amino-terminal mitochondrial membrane-binding domain of the somatic HK1 is subject to proteolysis (3, 5). Although the GCS domain does not resemble the mitochondrial membrane-binding domain, the specific degradation of the endogenous GCS domain also has been observed, even when sperm extracts were prepared in the presence of protease inhibitors and when stored at –70 °C.2 In this study it appeared that the GCS domain was most labile when attached to the relatively small GFP domain or when attached to the larger full-length H domain plus the GFP. The fact that the GCS/tH construct seemed to be targeted to the ER and translocated to the plasma membrane efficiently and that it was subject to the least amount of proteolysis dictated the subsequent design of additional constructs to include only the amino-terminal half of the hexokinase sequence.

The SA Domain of the Germ Cell-specific Protein, HK1-SA, Was Proteolytically Processed and May Be Involved in Mitochondrial Targeting—In addition to the HK1-sc transcript found to be expressed at the protein level in spermatozoa (12), two other germ cell-specific transcripts, HK1-sa and HK1-sb, are also present (10). Although no evidence for the HK1-SA protein was found using HK1-SA-specific antiserum in develop-

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<sup>3</sup> A. J. Travis, S. B. Moss, and G. S. Kopf, unpublished observations.
oping male germ cells or in mature sperm, we had suggested that this protein might avoid detection following translation if the unique amino-terminal region were proteolytically processed (12). Such processing would yield essentially the HK1-SC isoform as the mature product (Fig. 1). Therefore, constructs based on the HK1-sa sequence were generated. Regardless of whether the constructs began from the first in-frame ATG (SA/GCS/tH) or from the second in-frame ATG (tSA/GCS/tH), these constructs targeted to the ER in greater than 95% of the cells showing successful transfection and fusion protein expression (results not shown, but the appearance of transfected cells was indistinguishable from that shown in Fig. 3). However, in the remaining subpopulation of cells expressing the fusion protein, a pattern consistent with mitochondrial targeting was observed in addition to ER localization (Fig. 5). Immunoblots of cells transfected with constructs beginning at either possible initiator methionine revealed that processing of the unique SA amino-terminal domain had occurred to a large extent (Fig. 6). When probed with anti-GCS, a closely spaced doublet was seen in lanes containing tSA/GCS/tH and SA/GCS/tH, but only the upper band in this doublet was detected with anti-SA. When constructs tSA/GCS and SA/GCS were probed with anti-GCS, two bands in each lane were also observed, and the lower band co-migrated with construct GCS. Again, only the upper band was detected when probed with anti-SA. These results indicated selective loss of the SA region and indicated that this processing took place between the second in-frame ATG and the beginning of the GCS domain.

To investigate the potential role of the SA domain in mitochondrial targeting, constructs based on the SA domain alone, tSA and SA, or the SA and GCS domains together, tSA/GCS and SA/GCS, were created (Fig. 1). These constructs all displayed a diffuse pattern of fluorescence in both the cytoplasm and nucleus, a pattern consistent with GFP alone (results not shown). Immunoblots of cells transfected with these smaller constructs revealed that proteolytic processing to the GFP domain alone occurred at a much higher rate in these constructs than that seen with the larger constructs (results not shown). Therefore the diffuse pattern of fluorescence observed with these constructs cannot be distinguished from that of GFP alone, and results with these smaller constructs based on the SA domain were not interpretable.

The SB Domain of the Germ Cell-specific Protein, HK1-SB, Does Not Contain Intrinsic Targeting Information—As is the case with the HK1-SA isoform, the HK1-SB isoform is not detected in developing male germ cells or mature spermatozoa (12). However, the inability to detect this isoform might be due to a post-translational modification that would mask the epitope recognized by the anti-SB antiserum (12). To test whether the SB domain contained targeting information, constructs tH/SB and GCS/AH/SB (Fig. 1), based upon the HK1-SB isoform, were generated. Both the tH/SB and tH constructs showed a diffuse cytoplasmic pattern of fluorescence, and GCS/
tH/SB targeted to the ER (results not shown, but appearance of transfected cells was indistinguishable from that shown in Figs. 2D and 3C, respectively). The SB domain was found to confer no specific targeting information in this somatic cell system, whether it was tested in constructs that contained or were devoid of the GCS domain.

**The Carboxyl-terminal 10 Amino Acids of the GCS Domain Contain a Novel ER-targeting Motif**—The amino-terminal GCS domain, which targeted fusion proteins to the ER and plasma membrane, contains no documented ER-targeting motifs, nor is it hydrophobic in nature. Based on MacVector analysis (MacVector, Oxford Molecular Group, Beaverton, OR), the final 10 amino acid residues of the GCS domain were predicted to have a high surface probability, suggesting that they might be involved in protein-membrane or protein-protein interactions. To pinpoint the specific amino acid residues that are critical for ER targeting, a mutational analysis of the GCS domain was undertaken. A deletional mutant was made in which the first 14 amino acids of the GCS region were removed. This deletional mutant, mGCS(del1–14)/tH, continued to target to the ER, although some disruption of the ER was noted, and little or no fluorescence was seen at the plasma membrane (Fig. 7, A and B). The results obtained with this construct demonstrated that the ER-targeting information was contained within the last 10 amino acids of the GCS domain.

Although containing no obvious targeting motifs, these 10 residues have homology to the GCS domain of the human germ cell-specific HK. Upon further inspection, two groups of amino acids were recognized that bore some resemblance to di-basic and poly-proline motifs that have been implicated in membrane and protein interactions. The sequence KIR resembles a potential di-basic motif (18), although it differs from established ER-targeting motifs by its distance from the amino terminus. However, mutation of the lysine to asparagine or the arginine to glutamine did not prevent targeting to the ER or plasma membranes when performed either singly or in combination (Fig. 7C). Mutations to alanines of either the first proline alone (Fig. 7D), or the second and third prolines together (results not shown), resulting in the disruption of a potential
targeting. Also targeted to the ER, indicating that this proline was not critical for targeting, even though ER processing or stability might have been perturbed, an additional mutant was generated to test for the possibility that the ER-targeting motif might involve several of these amino acids as the region encoding the ER-targeting motif. The degree of homology between the last 10 amino acids increases if one considers a single amino acid shift between the two amino acid sequences (after Mori et al.; Ref. 11). Identity is denoted by a solid line, whereas conserved amino acid substitutions are denoted by dashed lines.

Poly-proline motif (19, 20), also did not prevent targeting of these constructs to the ER. An additional mutant construct was generated, altering the leucine, threonine, and glutamic acid to proline, alanine, and glutamine, respectively. Two of these three amino acids have homology to the human GCS domain (Fig. 7E and Ref. 11), and the side chain of threonine offers the potential for interaction through hydrogen bonding. As seen with the other mutants, this construct also did not revert the pattern of targeting back to the diffuse pattern characteristic of the GFP alone or of the HK sequence without the GCS or mitochondrial membrane-binding domain. Rather, the fusion protein was still targeted to the ER (Fig. 8A).

Similar to that seen with the mGCS(del1–14)/tH construct, the aforementioned point mutant constructs yielded ER and plasma membrane targeting in transfected cells. Yet this targeting was not entirely normal, in that when fields of cells were viewed at increasing times following transfection, the population of cells having a disrupted ER localization appeared to increase (Fig. 8). Fluorescence became concentrated in foci, or vesicles, that were distributed throughout the cell and that no longer appeared to be part of the ER or the ER/Golgi continuum. At the latest time points with many of these constructs, the vesicles seemed to accumulate in the cell periphery (Fig. 8D).

Because all of the mutants tested thus far retained ER targeting, even though ER processing or stability might have been perturbed, an additional mutant was generated to test for the possibility that the ER-targeting motif might involve several of the amino acids that were tested individually or in small groups. Amino acids to be altered in this “combination” mutant were chosen based upon homology to residues in the human GCS domain (11). All cells transfected with construct mGCS(P15A,K16N,R18Q,L21P,T22A,E23Q)/tH exhibited a diffuse pattern of fluorescence throughout the cytoplasm. The fact that fluorescence was not observed in the nucleus indicated that the fluorescence was not due to GFP alone but rather to a construct containing HK sequence. The data shown in this figure were gathered using the mGCS(L21P,T22A,E23Q)/tH construct but are representative of all the point and deletional mutant constructs tested. It should be noted that examples of both a normal and a disrupted ER pattern of fluorescence could be found at each time point, but the figures shown represent the typical pattern of fluorescence for that time point.

DISCUSSION

In highly polarized cells, the correct targeting of proteins to defined structures or compartments is critical for localizing specific functions to limited regions of the cell. In spermatozoa, the head is the carrier of the paternal genetic material, as well as being the region of the cell responsible for interacting first with the zona pellucida, the extracellular matrix of the oocyte, and then with the plasma membrane of the oocyte. The flagel-
lum is highly specialized to provide the sperm with progressive motility and is itself subdivided into different sections; the midpiece is the only region of the sperm that contains mitochondria, and both the midpiece and principal piece contain cytoskeletal scaffolding proteins that surround the axoneme. Our previous finding that one germ cell-specific isozyme of HK1 localizes to the membranes of the head, the mitochondria of the midpiece and the fibrous sheath of the flagellum (12) raised the question of how one protein can be targeted to such distinct biochemical compartments within a single cell type.

Because no lines of germ cells exist that are permissive for morphological differentiation into spermatozoa in culture, constructs containing different domains of the germ cell-specific HK1 transcripts HK1-sa, HK1-sb, and HK1-sc were linked to a carboxyl-terminal GFP marker and transfected into a somatic cell line deficient in HK. Although this system would not permit the study of targeting to the fibrous sheath (a structure that has no analogs in other cell types), it allows for the study of interactions with cell membranes, mitochondria, and other cytoskeletal components.

We found that the addition of the GCS domain, when expressed in this somatic cell system, was both necessary and sufficient to target HK-GFP fusion proteins to the ER and the plasma membrane. The targeting of this fusion protein to the ER and plasma membranes is consistent with the localization of the endogenous protein to the membranes of the sperm head (12). Moreover, the appearance of the fusion proteins in the ER and plasma membranes demonstrated that the targeting mechanism was not germ cell-specific and that this heterologous system possessed the necessary cellular machinery for ER localization and translocation to the plasma membrane.

Targeting of the GCS domain to the ER and plasma membrane occurred despite the absence of a hydrophobic signal sequence. Precedent exists for plasma membrane or extracellular localization of proteins that are not hydrophobic in nature. For example, a muscle lectin has been shown to be “externalized” from cells by means of a novel secretory mechanism (21). However, the model suggested for this particular mechanism requires that it maintains a cytosolic localization in vesicles that are themselves released from the cell and then opened (21). In contrast, the endogenous HK1-SC protein is not soluble. Indeed, at least some of the protein has the biochemical characteristics of an integral membrane protein, in that it is not solubilized by conditions that dissociate most peripheral membrane proteins, nor is it solubilized by conditions that dissociate somatic HK from mitochondria (12, 14). The fusion proteins generated in this study appeared to be associated with the membranes of vesicles that integrated into the plasma membrane, so it is unlikely that they were externalized by the mechanism used by the muscle lectin.

Most proteins are targeted to the ER by a signal recognition particle-mediated pathway that recognizes and binds a hydrophobic signal sequence on the amino terminus of a protein (for review see Ref. 22). As noted, the GCS domain does not contain a hydrophobic region. Rather, the results of our mutational analysis demonstrated that a novel motif, PKIRPPLTE (with residues in bold print appearing to be of particular importance), was responsible for ER targeting. Whether this motif operates by being bound in the signal recognition particle-mediated pathway or is processed through a signal recognition particle-independent pathway is unknown. Similarly, whether membrane targeting takes place through a direct interaction of this motif with the membranes or secondarily through the interaction of this motif with another membrane protein will form the basis of future study.

Mutations of individual amino acids within this novel motif led to a disruption of the ER, characterized by an increased “vesiculation” and loss of the normal reticular appearance. Based on the appearance of cells expressing these mutant constructs at various times after transfection, it seems that initial ER targeting was intact but that either the ER membranes became fragmented into vesicles or that processing through the Golgi lost directionality and normal vesicles dispersed throughout the cell. A similar result was seen when the 14 amino acids amino-terminal to the targeting motif were removed, and the strong conservation between the mouse and human sequences of the amino-terminal 7 residues within the GCS domain suggests an important role for those residues. These results suggest that those amino-terminal amino acids might also interact with the ER membranes in some way to influence processing to the Golgi and plasma membrane or that they might interact with other proteins that contribute to the normal ultrastructural morphology of the ER.

The time course data with the mutant constructs also revealed that at later times after transfection the majority of the disrupted vesicles aggregated at the cell periphery underlying the plasma membrane. It has been reported that somatic HK can translocate to the cell cortex of activated rat macrophages (23), presumably through an interaction with cytoskeletal elements. Whether our fusion proteins congregated in the cell periphery because they were still able to interact with specific components of the plasma membrane or whether there were secondary interactions with cytoskeletal elements or some other translocating mechanism is unknown.

When the GCS domain was linked directly to GFP or to the full-length HK sequence, proteolytic removal of the GCS domain occurred at higher levels than when the GCS domain was linked to a half-length HK sequence. One possible explanation for this trend is that the additional HK sequence provided limited stability against protease activity. Proteolytic degradation of amino-terminal domains was not limited to the GCS region. Fusion proteins based on the HK1-sa sequence, regard-
less of which initiator methionine was used, showed the specific removal of the SA domain. That this processing occurred in a heterologous system suggested that the protease was not germ cell-specific. The only clear instances of mitochondrial targeting observed in this study were seen with fusion proteins containing the SA domain. It is tempting to speculate that the proteolytic removal of the SA domain immediately amino-terminal to the GCS domain might regulate a chimeric targeting motif. Precedent for such chimeric targeting motifs that result in either ER or mitochondrial localization dependent upon motif processing exists (24). However, the susceptibility of fusion proteins containing the SA domain linked to GFP either directly (tSA and SA) or with an intervening GCS domain (tSA/GCS and SA/GCS) to endogenous proteolytic degradation precluded conclusions on this matter.

Recently, the localization of a protein kinase A-anchoring protein (D-AKAP1) to either the mitochondria or to the ER was shown to result from the alternative splicing of different amino-terminal domains (25). This finding is noteworthy in two ways. First, it provides an alternative method of targeting a given protein to two different organelles. Interestingly, the HK1-sa, HK1-sb, HK1-sc, and somatic HK1 transcripts also arise from alternative splicing of a single gene (26). Should the proteolytic processing of the SA domain seen in the present study be involved in the targeting of that isoform in germ cells, then the targeting of HK1 in germ cells would be regulated by alternative splicing as well as by the processing of targeting domains. This added complexity would also suggest that the GCS domain, which is included in both transcripts, might be necessary in protein function or at least to maintain an association with a given membrane, be it ER, plasma membrane, or outer mitochondrial membrane.

The second interesting comparison with the D-AKAP1 findings relates to the nature of its mitochondrial membrane-binding domain. This domain is similar in amino acid sequence to the mitochondrial membrane-binding domain of the somatic HK1 (25). It is intriguing that alternative splice variant domains responsible for the mitochondrial localization of two proteins that can target to multiple organelles have such similarity. Whether this similarity in mitochondrial targeting domains is the result of convergent evolution or a common ancestor for that domain is unknown. D-AKAP1 was originally characterized in spermatids as S-AKAP84, found in the mitochondria of the midpiece, and was suggested to localize to that organelle by means of an amino-terminal targeting motif (27). Other AKAPs localize to the fibrous sheath of the principal piece (28), and we have suggested that the co-localization of an AKAP and glycolytic enzymes in the fibrous sheath might allow ATP to be produced at the point where it is needed to be used in the regulation of sperm motility (12). Given the critical nature of the flagellar ultrastructure in sperm function and the absence of translocating mechanisms in mature spermatozoa, the assembly of the flagellum and the inherent targeting of its components in germ cells takes on added importance.

Along with differences in sequence, fusion protein targeting to the mitochondria seen with constructs tSA/GCS/H and SA/GCS/H might be dependent upon time of expression relative to the cell cycle. Endogenous HK1-sa is expressed in meiotic germ cells, whereas HK1-sc is expressed post-meiotically (10, 14). The possibility that only the subset of cells transfected at a specific stage of the cell cycle would show mitochondrial localization might explain why mitochondrial targeting was seen in such a limited number of cells. Alternatively, the fact that somatic mitochondria are substantially different from sperm mitochondria (29), might lower the efficiency with which the germ cell-specific domains interact with the somatic mitochondria. Lastly, an example of a protein being targeted to the Golgi in a somatic tissue and in early stages of germ cell development, but to the midpiece of mature spermatozoa, also exists (30). This finding suggests that targeting to the ER can precede targeting to mitochondria in germ cells.

Germ cells possess organelles such as the fibrous sheath, which is not found in somatic cells. Moreover, even organelles common to germ and somatic cells (e.g. mitochondria) differ in details of their structure and function. Thus it is evident that studies such as the present one, employing a heterologous system for expression of germ cell-specific proteins, has its limitations. Nonetheless, such studies provide a feasible approach for detecting sequence elements that may play a targeting role in germ cells. In future work, the significance of such elements in germ cell differentiation in vivo will be explored using transgenic animal models that allow expression in the appropriate cellular environment and temporal pattern.

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