MOLECULAR AND CELLULAR BIOLOGY, July 1995, p. 3507–3515 Vol. 15, No. 7
0270-7306/95/$04.00+0

Myristoylation and Differential Palmitoylation of the *HCK* Protein-Tyrosine Kinases Govern Their Attachment to Membranes and Association with Caveolae

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Received 22 December 1994/Returned for modification 21 February 1995/Accepted 6 April 1995

The human proto-oncogene *HCK* encodes two versions of a protein-tyrosine kinase, with molecular weights of 59,000 ($p59^{hck}$) and 61,000 ($p61^{hck}$). The two proteins arise from a single mRNA by alternative initiations of translation. In this study, we explored the functions of these proteins by determining their locations within cells and by characterizing lipid modifications required for the proteins to reach those locations. We found that $p59^{hck}$ is entirely associated with cellular membranes, including the organelles known as caveolae; in contrast, only a portion of $p61^{hck}$ is situated on membranes, and none is detectable in preparations of caveolae. These distinctions can be attributed to differential modification of the two *HCK* proteins with fatty acids. Both proteins are at least in part myristoylated, $p59^{hck}$ more so than $p61^{hck}$. In addition, however, $p59^{hck}$ is palmitoylated on cysteine 3 in the protein. Palmitoylation of the protein requires prior myristoylation and, in turn, is required for targeting to caveolae. These findings are in accord with recent reports for other members of the *SRC* family of protein-tyrosine kinases. Taken together, the results suggest that *HCK* and several of its relatives may participate in the functions of caveolae, which apparently include the transduction of signals across the plasma membrane to the interior of the cell.

The proto-oncogene *HCK* is one of nine members of a multigene family, for which the closely related proto-oncogene *SRC* has served as the archetype (10). These genes encode protein-tyrosine kinases (PTKs) with a number of characteristic structural features, including a catalytic domain occupying the carboxy-terminal half of the proteins; smaller domains known as SH2 and SH3, which permit specific interactions with other proteins; and an amino-terminal sequence of ca. 70 amino acids that is different in each member of the family. In addition, all of the *SRC* family PTKs are myristoylated on a glycine residue at their amino termini (53).

HCK encodes two forms of protein, with molecular weights of 56,000 and 59,000 in mouse cells (38) and of 59,000 ($p59^{hck}$) and 61,000 ($p61^{hck}$) in human cells (see below). In both species, the two isoforms arise from alternative initiations of translation on a single mRNA, the smaller at an AUG and the larger at a CUG (Fig. 1) (38, 57). As a result, the larger protein in both species contains an additional 21 residues at its amino terminus. The functional significance of this difference is not known.

The PTKs encoded by the *SRC* gene family are generally associated with membranous organelles, and myristoylation is typically required for this association (53). Examples reported include specialized portions of the plasma membrane known as adhesion plaques (11, 26, 55, 60), late endosomes (12, 27), synaptic vesicles (34), chromaffin granules (21, 49), granules and secretory vesicles of platelets (52), and most recently, the organelles known as caveolae (64, 67). Frequently, the kinases have been found tightly anchored to the cytoplasmic face of the membrane (53). It is suspected that the varied subcellular locations of *SRC* family PTKs connote differences in biochemical functions, but there is little evidence at present to support this suspicion.

Relatively little is known about the functions of the two proteins encoded by *HCK*. The gene is expressed principally in myelomonocytic hematopoietic cells and in B lymphocytes (23, 31, 51, 86). Expression is at a relatively low level in the immature precursors to granulocytes and macrophages but rises sharply when these cells differentiate to mature forms (23, 28, 32, 51). Thus, it has been presumed that the *HCK* proteins must serve some role in either the differentiation or the mature function of leukocytes, and there is some provisional evidence to support this view (16, 19, 39, 79).

It is generally believed that *SRC* family PTKs serve to transduce signals from transmembrane proteins to the interior of the cell (63). There are hints that this may be true of the *HCK* products. In particular, activation of the *HCK* PTK may help mediate the cellular effects of lipopolysaccharide (LPS) (16, 73) and cytokines (17, 35). In these settings, the *HCK* kinase may be serving as signal transducer for a transmembrane protein that does not itself possess an enzymatic activity.

Here we report our efforts to explore the function of human $p59^{hck}$ and $p61^{hck}$ by localizing these proteins within the cell. We find that the two proteins are distributed differently between the cytoplasm and membranous organelles. First, the association of $p59^{hck}$ with membranes is more complete than is that of $p61^{hck}$. Second, ca. 20% of $p59^{hck}$ is associated with caveolae, and this association can be attributed to palmitoylation of the protein on cysteine 3. In contrast, $p61^{hck}$ is neither palmitoylated nor associated with caveolae. These findings are in accord with recent reports for other members of the *SRC* family of PTKs (59, 67). Thus, $p59^{hck}$ and several of its relatives may participate in the functions of caveolae, which apparently include the transduction of signals across the plasma membrane (2, 3, 36).

MATERIALS AND METHODS

Construction of mutant *HCK* alleles. The *HCK* cDNAs that were constructed are illustrated in Fig. 1. The *HCK* cDNA (51) that encodes both $p59^{hck}$ and

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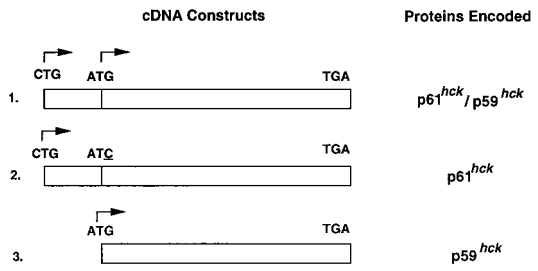

FIG. 1. Construction of *HCK* alleles. Standard cloning techniques were used to construct murine retrovirus vectors encoding both $p61^{hck}$ and $p59^{hck}$ (construct 1), $p61^{hck}$ (construct 2), and $p59^{hck}$ (construct 3). The *HCK* alleles were introduced into NIH 3T3 cells as described in Materials and Methods.

$p61^{hck}$ was subcloned as a *Sal*I fragment into plasmid pSP72 (Promega) and was used to generate any further constructs. To generate a $p59^{hck}$-specific cDNA, the parental *HCK* cDNA was digested with *Nar*I, made blunt, digested with *Sal*I, and then ligated into pSP72. A $p61^{hck}$-specific cDNA was generated by PCR using an oligonucleotide that started at the *Nar*I site and converted the ATG initiation codon to ATC and an antisense oligonucleotide encompassing the unique *Hin*dIII site within the *HCK* cDNA. The resulting PCR fragment was digested with *Nar*I and *Hin*dIII and used to replace the corresponding fragment in the $p59^{hck}$ cDNA. The *HCK* cDNA was digested with *Sal*I and *Nar*I, and the 5′ 72-bp fragment encompassing the CTG initiation codon was purified and ligated into the p59ATC cDNA to generate $p61^{hck}$ cDNA. To verify that all of the resulting cDNAs encoded the appropriate *HCK* proteins, in vitro transcription-translation was performed with commercially available reagents (Promega), and the resulting [^{35}S]methionine-labeled translation products were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Each of the resulting pSP72-*HCK* constructs was digested with *Xho*I and *Cla*I to excise the entire cDNAs and subcloned into a replication-defective murine retrovirus vector, LNCX (kind gift of Dusty Miller) (42), that had been modified to contain unique *Xho*I and *Cla*I sites. The vectors encode resistance to G418, allowing selection of infected cells, and expression of the *HCK* alleles is driven by the cytomegalovirus promoter.

The other N-terminal *HCK* mutants (see Fig. 4B) were all generated by the following PCR strategy. Sense oligonucleotides with a 5′ unique *Xho*I site encompassing the first five amino acids of coding sequence containing the desired amino acid mutations depicted in Fig. 4B were synthesized. The antisense oligonucleotide encompassing the unique *Hin*dIII site was synthesized and used in PCRs with the various sense oligonucleotides. The resulting PCR fragments were digested with *Xho*I and *Hin*dIII and used to replace the corresponding fragment within the LNCX-*HCK* vectors described above. To achieve a higher degree of expression of the cDNA constructs encoding wild-type p61 and mutant p61G3C, the 5′ sense oligonucleotides replaced the CUG with the more conventional AUG initiation codon in the context of an appropriate Kozak sequence (30). All constructs were verified by complete nucleotide sequence analysis.

Antibodies. The rabbit polyclonal antiserum to *HCK* proteins used for immunoprecipitations and Western blot (immunoblot) analyses was raised against a glutathione *S*-transferase fusion protein encompassing amino acids 34 to 198 of the human *HCK* protein (32). The *HCK* antiserum recognized both human *HCK* proteins ($p59^{hck}$ and $p61^{hck}$) but did not recognize the murine *HCK* proteins. In addition, the *HCK* antiserum did not recognize any other *SRC* family PTKs. The anticaveolin monoclonal antibody was purchased from Transduction Laboratories.

Cell culture and virus production. U937 cells (American Type Culture Collection) were maintained in RPMI 1640 supplemented with 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 2 mM glutamine, 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, and 5×10^{-5} M β-mercaptoethanol. To induce differentiation to the more mature macrophage phenotype, cells were washed, suspended in fresh medium containing 12-*O*-tetradecanoylphorbol-13-acetate (TPA) at a concentration of 20 ng/ml, and incubated for 48 to 76 h.

The established murine fibroblast cell line C7-3T3 is a clonal derivative of cell line NIH 3T3 (kindly provided by E. T. Liu) and was maintained in Dulbecco's modified Eagle minimal medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum. Replication-defective helper-free virus stocks were produced by transfection of Ψ2 cells with the appropriate LNCX-based plasmids as described previously (41). C7-3T3 cells were infected with these virus stocks as described previously (41), and pools of infected cells were selected by virtue of their resistance to the drug G418 (GIBCO-BRL).

Metabolic labeling. U937 cells (1×10^7 to 2×10^7 cells in a 100-mm-diameter plate) were incubated for 30 min to 1 h in methionine-free RPMI containing 10% dialyzed fetal bovine serum and then incubated for 5 h in the same medium containing 200 μCi of L-[^{35}S]methionine (>1,000 Ci/mmol; Amersham) per ml in a total volume of 5 ml. C7-3T3 cells were starved and labeled as described above in methionine-free DMEM.

For metabolic labeling with fatty acids, U937 cells (1×10^7 to 2×10^7 cells in a 100-mm-diameter dish) were labeled in RPMI 1640 containing 10% dialyzed fetal bovine serum and 500 μCi of 9,10-[^{3}H]myristic acid (39.3 Ci/mmol; DuPont NEN) per ml for 18 h at 37°C. C7-3T3 cells were labeled with myristic acid as described above in DMEM. To label C7-3T3 cells with palmitic acid, confluent cells in two to four 100-mm-diameter plates were labeled in DMEM containing 10% dialyzed fetal calf serum, 5 mM pyruvic acid, 40 mM nonessential amino acids, and 500 μCi of 9,10-[^{3}H]palmitic acid (39.0 Ci/mmol; DuPont NEN) per ml in a total volume of 4 ml per plate for 2 to 4 h at 37°C. Palmitic acid was prepared by being dried under nitrogen gas to a final concentration of 50 mCi/ml.

Preparation of cell lysates and Western blotting. Cells were washed two to three times in cold phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} and lysed on ice in Nonidet P-40 (NP-40) lysis buffer, composed of 50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 100 mM NaF, 10% glycerol, 1% NP-40 (protein grade; Calbiochem), 1 mM Na_2VO_4, 1 mM Pefabloc (Boehringer Mannheim), 10 μg of aprotinin per ml, and 10 μg of leupeptin per ml. Insoluble material was removed by centrifugation at 12,000 × *g* for 10 min at 4°C. The protein concentration of the soluble material was estimated by using a bicinchoninic acid protein assay kit (Pierce, Rockford, Ill.). Laemmli's sample buffer was added to the cleared lysate and heated to 100°C for 10 min. Equal amounts of total cell protein were loaded onto a 10% polyacrylamide gel. Following electrophoresis, proteins were transferred to nitrocellulose (Schleicher & Schuell) for 1 h at 1.5 mA/cm^2, using a semidry transfer apparatus (E & K Scientific Products Inc., Saratoga, Calif.). The nitrocellulose filters were blocked by incubation in Tris-buffered saline (5 mM Tris, 135 mM NaCl, 5 mM KCl) containing 5% nonfat dry milk, 0.5% NP-40, and 0.1% Tween 20 for 1 h at room temperature followed by a 1-h incubation in primary antibody diluted in blocking buffer. The filters were then washed extensively in Tris-buffered saline containing 0.5% NP-40 and 0.1% Tween 20 before incubation for 30 min with either donkey anti-rabbit–horseradish peroxidase or sheep anti-mouse–horseradish peroxidase conjugate (Amersham, Arlington Heights, Ill.), each diluted 1:10,000 in blocking buffer. The filters were then washed as described above and developed by using an enhanced chemiluminescence substrate (Amersham).

Immunoprecipitations. Labeled cells were lysed in NP-40 lysis buffer as described above. For L-[^{35}S]methionine-labeled extracts, equal amounts of trichloroacetic acid (TCA)-precipitable radioactivity were used, whereas for fatty acid labelings, the entire cell lysate from a single labeling experiment was used. Cell-free lysates were precleared by incubation with preimmune rabbit serum and protein A-Sepharose for 1 h at 4°C. After the preimmune protein A complexes were removed by centrifugation, the cleared lysates were incubated with an excess of polyclonal anti-*HCK* antiserum for 1 h at 4°C, protein A-Sepharose was added, and the mixture was incubated for an additional 30 min. After the immune complexes were washed several times in NP-40 lysis buffer, the L-[^{35}S]methionine- and 9,10-[^{3}H]myristic acid-labeled samples were boiled in Laemmli's buffer. The 9,10-[^{3}H]palmitic acid-labeled samples were heated for 5 min at 80°C in Laemmli's buffer without reducing agents and allowed to cool, and then dithiothreitol was added to a final concentration of 2 mM. All samples were analyzed on an SDS–10% polyacrylamide gel; after treatment of the gel with Amplify (NEN DuPont) according to the manufacturer's specifications, autoradiography was performed. The stoichiometry of myristoylation of the *HCK* proteins was calculated from densitometry on data from several independent labeling experiments, using the formula ([^{3}H]myristic acid $p61^{hck}$/[^{3}H]myristic acid $p59^{hck}$)/([^{35}S]Met $p61^{hck}$/[^{35}S]Met $p59^{hck}$).

Subcellular fractionations. Crude cell extracts prepared by Dounce homogenization of hypotonically swelled cells were fractionated into cytosol and membrane fractions by differential centrifugation. Briefly, either labeled or unlabeled cells were washed twice in ice-cold PBS without Ca^{2+} and Mg^{2+} and then suspended in hypotonic buffer (10 mM Tris-HCl [pH 8.0], 10 mM KCl, 1 mM EDTA, 0.1% β-mercaptoethanol, 1 mM Pefabloc, 10 μg each of aprotinin and leupeptin per ml). Cells were allowed to swell for 20 min on ice and then lysed with 20 strokes (C7-3T3 cells) or 50 strokes (U937 cells) in a Dounce homogenizer. Nuclei and any remaining intact cells were removed by low-speed centrifugation (10 min at 1,000 × *g*). Cytosol and membrane fractions were prepared from the postnuclear supernatant by high-speed centrifugation (50,000 × *g*) for 1 h at 4°C. The supernatant (cytosol) fraction was removed, and the membranes were resuspended in the hypotonic lysis buffer containing 150 mM NaCl, 1% NP-40, and 0.2% SDS. The cytosol fraction was adjusted to the same final concentrations of NaCl, NP-40, and SDS. Immunoprecipitations with anti-*HCK* antiserum and subsequent SDS-PAGE analysis were carried out as described previously.

For Western blot analysis of the cytosolic and membrane fractions, each sample was adjusted to 10% TCA and incubated on ice for 20 min. The samples were spun at 10,000 × *g* for 20 to 30 min at 4°C, and Laemmli's buffer containing 0.2 M Tris base was added to the pellets. The pellets were resuspended, boiled for 10 min, and then subjected to SDS-PAGE and Western blotting as described above. Densitometry on Western blots was used to determine the relative subcellular distribution of wild-type and mutant $p61^{hck}$.

Isolation of caveolae. Triton-insoluble complexes containing caveolae were prepared by an isolation procedure originally described by Brown and Rose (5)

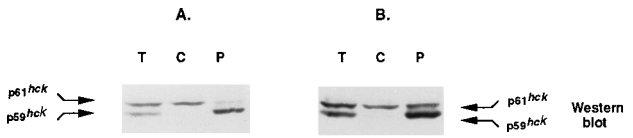

FIG. 2. Subcellular distribution of $p59^{hck}$ and $p61^{hck}$ in the human myelomonocytic cell line U937. Undifferentiated (A) or differentiated (B) U937 cells were lysed in hypotonic lysis buffer by Dounce homogenization. Crude extracts (T) were fractionated into cytosolic (C) and particulate (P) membranes by centrifugation at 50,000 × *g*. Western blot analysis using a rabbit polyclonal antiserum to human *HCK* was performed on fractions derived from equal numbers of U937 cells as described in Materials and Methods. The positions of $p59^{hck}$ and $p61^{hck}$ are indicated.

and recently modified by Sargiacomo et al. (64). Briefly, 5×10^7 U937 cells or, in a 150-mm-diameter plate, confluent C7-3T3 cells expressing the various *HCK* alleles described in this report were lysed in 2 ml of morpholineethanesulfonic acid (MES)-buffered saline (MBS; 25 mM MES [pH 6.5], 150 mM NaCl, 1 mM Pefabloc, 1 mM Na_2VO_4, 10 μg each of aprotinin and leupeptin per ml) containing 1% Triton X-100, adjusted to 40% sucrose, and placed on the bottom of an ultracentrifuge tube. A 5 to 30% linear sucrose gradient was formed above the samples in MBS and then centrifuged in an SW41 rotor (Beckman) at 37,000 rpm for 16 h at 4°C. In initial experiments, 1-ml fractions were collected across the entire gradient, and the proteins were precipitated by the addition of TCA and then analyzed by SDS-PAGE and Western blotting as described above. Alternatively, the Triton-insoluble complexes were collected by removing the opaque band corresponding to caveolae (migrating at approximately 10 to 20% sucrose) diluted 1:4 in MBS and pelleted in an SW41 rotor at 37,000 rpm for 1 h at 4°C. The precipitated proteins (caveolae) were suspended in MBS, Laemmli's sample buffer was added, and the samples were heated at 100°C for 10 min. The Triton-soluble fraction was collected by harvesting the bottom 2 ml of the gradient, with care to avoid the insoluble pellet below the 40% sucrose, and precipitating the total protein with TCA. Both caveolae (Triton-insoluble) and triton-soluble protein samples were subjected to SDS-PAGE (10% polyacrylamide gel) and Western blot analysis as described above.

RESULTS

The two products of human *HCK* are distributed differently between the cytoplasm and membranous organelles. In unpublished work, we have found that a single cDNA representing the mRNA of human *HCK* can give rise to both $p59^{hck}$ and $p61^{hck}$. We then used site-directed mutagenesis to demonstrate that $p59^{hck}$ arose from initiation of translation at an AUG, whereas $p61^{hck}$ was initiated from a CUG 21 codons upstream (Fig. 1 and data not shown). These findings are similar to those reported previously for mouse *HCK* (38) and thus are not reported in detail here.

The two products of mouse *HCK* have distinctive subcellular distributions in a variety of hematopoietic cells (38). The smaller protein was found exclusively in a crude membrane fraction, whereas the larger protein was present in both membranes and cytosol. We have made similar observations with the products of human *HCK* (Fig. 2). In lysates of the myelomonocytic cell line U937, $p59^{hck}$ was found only in the membrane fraction, whereas $p61^{hck}$ was distributed between membranes and cytosol (Fig. 2A). Differentiation of the cells by application of TPA did not change this distribution (Fig. 2B), although the amounts of both *HCK* proteins rose substantially, in accord with previous studies of *HCK* RNA in human cells (32, 51).

We also assessed the kinase activities of the two human *HCK* proteins by means of autophosphorylation following immunoprecipitation. The distribution of kinase activities between cytosol and membranes appeared to mirror that of the two *HCK* proteins (data not shown). Differentiation of U937 cells caused an increase in the amounts of *HCK* kinase activity, corresponding to the increases in amount of protein (data not shown), but there was no effect on either the relative enzymatic activities of the two *HCK* proteins or the subcellular distribution of kinase activity. Similar results were obtained with two other lines of human myelomonocytic cells (HL-60 and ML-1) and a line of B-lymphoid cells (IM-9) (data not shown).

Myristoylation of human *HCK* proteins is not the sole determinant of subcellular distribution. The PTKs encoded by *SRC* family genes are myristoylated on an amino-terminal glycine, and this modification is required for attachment of the proteins to membranes (53, 54). The myristoylation is dictated by consensus features within the first seven amino-terminal residues of the proteins (24, 76, 77) (see Fig. 4A). Since an appreciable fraction of the larger form of both mouse and human *HCK* protein was found in the cytosol, the possibility arose that this fraction of the protein is not myristoylated. A previous study with mouse cells reached the tentative conclusion that this was indeed the case, but the analyses were not definitive (38). We have not examined the issue directly with human *HCK* and find that the presence of $p61^{hck}$ in the cytosol cannot be explained entirely by a lack of myristoylation.

To examine the subcellular localization of the *HCK* proteins and to allow further study of the two forms independently, we created cDNA alleles of human *HCK* that expressed only one form or the other (Fig. 1). A retroviral vector was then used to express wild-type *HCK* and the two independent alleles in NIH 3T3 fibroblasts (see Materials and Methods for details). When expressed in NIH 3T3 cells, a wild-type *HCK* cDNA gave rise to $p59^{hck}$ and $p61^{hck}$ in a ratio resembling that found in hematopoietic cells, and the subcellular distributions of the two proteins were approximately as found before (data not shown). Moreover, the subcellular distributions did not change appreciably when the two forms of human *HCK* protein were expressed independently, nor were the results affected by different levels of expression (data not shown). We conclude that the distribution of human *HCK* protein among subcellular fractions in NIH 3T3 cells resembles that found in hematopoietic cells and proceeded to exploit this observation in further studies of how the proteins might be targeted to their subcellular locations.

We demonstrated that both forms of human *HCK* protein are myristoylated by labeling cells expressing wild-type *HCK* with [^{3}H]myristic acid. To assess the relative efficiency of myristoylation on different forms of *HCK* protein, samples of cells were labeled in parallel with [^{35}S]methionine. According to this analysis, the two forms of *HCK* protein were expressed in approximately equal amounts in either U937 cells (Fig. 3A; see also Fig. 5) or NIH 3T3 cells (data not shown), as found previously by Western blotting (Fig. 2). Similarly, both forms of protein were labeled with myristic acid (Fig. 3), although the labeling of $p61^{hck}$ was only 30% as efficient as that of $p59^{hck}$ in either U937 cells (Fig. 3A) or NIH 3T3 cells (Fig. 3B) (ascertained as described in Materials and Methods). We used treatment with neutral hydroxylamine and β-mercaptoethanol to authenticate the nature of the labeling (see below).

We then prepared subcellular fractions of the cells labeled with myristic acid (Fig. 3). As before, both $p59^{hck}$ and a portion of $p61^{hck}$ were found in the membrane fraction, but in contrast to the expectation from previous work (38), myristoylated $p61^{hck}$ was also found in the cytosol, in quantities approximately the same as those in the membrane fraction (Fig. 3). Similar results were obtained when the two *HCK* proteins were expressed together in either U937 or NIH 3T3 cells or independently in NIH 3T3 cells (Fig. 3). We conclude that the presence of $p61^{hck}$ in the cytosol cannot be attributed solely to the absence of myristoylation (we presume that the unmyristoylated fraction of $p61^{hck}$ would be consigned to the cytosol). These results prompted us to seek additional modifications

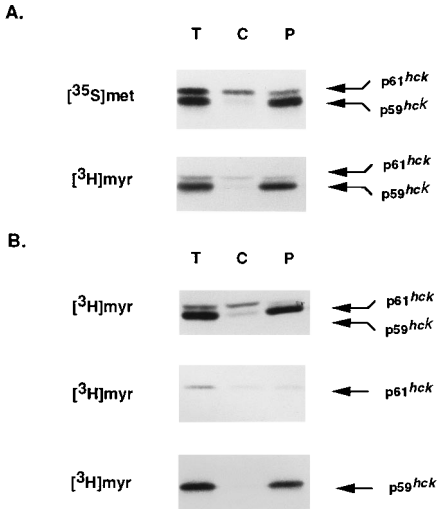

FIG. 3. Influence of myristoylation on subcellular localization of the *HCK* proteins. (A) U937 cells were metabolically labeled with L-[^{35}S]methionine ([^{35}S]met) or 9,10-[^{3}H]myristic acid ([^{3}H]myr). Following labeling, crude cell extracts (T) were fractionated into cytosolic (C) and particulate (P) membranes by differential centrifugation. *HCK* immunoprecipitates were prepared from fractions derived from equal numbers of cells. (B) NIH 3T3 cells expressing p59^{hck} and p61^{hck} (top row), only p61^{hck} (middle row), or only p59^{hck} (bottom row) were metabolically labeled with [^{3}H]myristic acid, and *HCK* immunoprecipitations were performed on fractionated cell extracts as described above. The samples were separated by SDS-PAGE, and fluorography was performed. The positions of p59^{hck} and p61^{hck} are indicated.

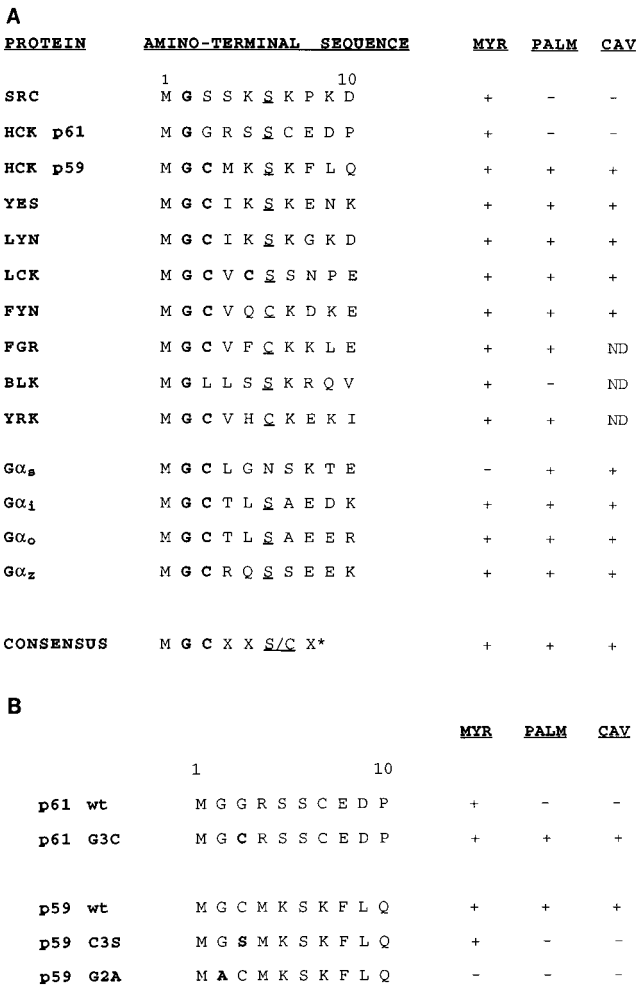

FIG. 4. Lipid modifications of the *SRC* family PTKs and the G_α subunits. (A) The N-terminal 10 amino acids for the *SRC* PTKs and several G_α subunits are indicated in the single-letter amino acid code. Proteins that have been shown or proposed to be modified (+) or not (−) by the fatty acids myristate (MYR) and palmitate (PALM) are indicated. Critical amino acids for the potential fatty acid modifications are in boldface or underlined. The association of the proteins with detergent-resistant complexes consistent with caveolae (CAV) is also indicated (ND, not determined experimentally). The minimal consensus sequence for the dual acylation by myristate and palmitate is shown, with X representing any amino acid and the X* position having some preference for certain amino acids (20, 24). This consensus sequence would also be predicted to be sufficient for localization to caveolae. (B) The N-terminal sequences of the *HCK* wild-type (wt) and mutant alleles are represented, with the amino acids that differ indicated in boldface. The lipid modifications and association with caveolae of the encoded products are indicated as described above. All references used to compile this figure are cited in the text.

that might contribute to the membranous attachment of *HCK* proteins.

The smaller of the human *HCK* proteins is palmitoylated. A growing variety of proteins are known to be palmitoylated (40, 54), and a number of these proteins are also myristoylated (1, 22, 29, 33, 44, 48, 59, 65, 67). Moreover, a compilation of amino-terminal sequences revealed consensus features shared by those proteins that are both myristoylated and palmitoylated (Fig. 4A). In these sequences, myristoylation occurs at the second residue (a glycine, exposed by removal of the amino-terminal methionine), and palmitoylation occurs at the third and/or fifth residues (cysteines). Members of the *SRC* family PTKs that lack cysteine at both the third and fifth residues are not palmitoylated (1, 29, 65) (Fig. 4A). The amino-terminal sequence of human p59^{hck} conforms to the requirements for palmitoylation, whereas the sequence of p61^{hck} does not (Fig. 4A). We pursued these contrasts by examining human *HCK* proteins for modification by palmitate.

We first examined NIH 3T3 cells expressing wild-type human *HCK* (Fig. 5). Labeling with [^{35}S]methionine revealed that the two forms of *HCK* protein were produced in approximately equal amounts. As before, both forms could be labeled with myristic acid, although once again, the labeling of p59^{hck} was more efficient than that of p61^{hck}. In contrast, palmitic acid labeled only p59^{hck}, as expected from the comparison of amino acid sequences described above.

We authenticated the nature of the modifications by chemical analysis of the linkages between the fatty acids and amino acids. Myristoylation involves a stable amide bond to glycine, whereas palmitate is linked to cysteine by means of a relatively labile thioester bond. Using treatment with either β-mercaptoethanol or neutral hydroxylamine, we found that the labeling of p59^{hck} with palmitic acid could be disrupted, whereas the labeling of various *HCK* proteins with myristic acid could not (data not shown).

Palmitoylation of *HCK* proteins requires myristoylation at the amino terminus and cysteine at residue 3. To explore the structural requirements for modification of *HCK* proteins with fatty acids, we constructed mutant alleles in cDNAs that created the following changes: cysteine 3 to serine in p59^{hck} (p59C3S), glycine 2 to alanine in p59^{hck} (p59G2A), and glycine 3 to cysteine in p61^{hck} (p61G3C) (Fig. 4B). The mutants were expressed independently by means of a retroviral vector in NIH 3T3 cells. In the course of this work, we discovered that mutation of the AUG to AUC (to create the p61^{hck} allele) substantially reduced the production of p61^{hck}. For purposes of the following experiments, it was necessary to augment production of p61^{hck} by replacing the CUG initiation codon with

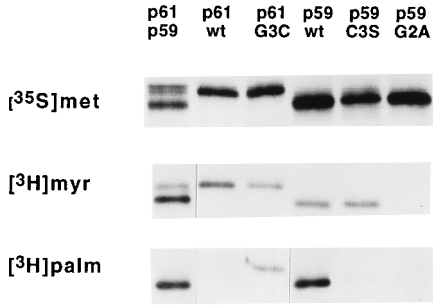

FIG. 5. Myristoylation and palmitoylation of wild-type (wt) and mutant *HCK* proteins. NIH 3T3 cells that were stably expressing various alleles of human *HCK*, as indicated above each lane, were metabolically labeled with either [^{35}S]methionine ([^{35}S]met; top row), 9,10-[^{3}H]myristate ([^{3}H]myr; middle row), or 9,10-[^{3}H]palmitate ([^{3}H]palm; bottom row). Following labeling, the cells were lysed in 1% NP-40 lysis buffer and the proteins were immunoprecipitated with a rabbit polyclonal antibody to *HCK*. All samples were resolved by SDS-PAGE followed by treatment of the gel in Amplify (NEN DuPont) before autoradiography.

AUG, along with an optimal upstream nucleotide sequence (data not shown). We used this configuration for the production of both wild-type and mutant p61^{hck} and achieved expression comparable to that found with the wild-type cDNA (Fig. 5).

We assessed the effects of these mutations on both myristoylation and palmitoylation (Fig. 5). As expected, p59G2A was not labeled with myristic acid, but the other mutations had negligible effects on this modification. The same mutation also eliminated palmitoylation, suggesting that myristoylation is a requirement for the additional modification by palmitate. The mutation p59C3S also eliminated palmitoylation but had no effect on myristoylation. Conversion of the third residue of p61^{hck} from glycine to cysteine (p61G3C) allowed this protein to be palmitoylated, albeit less efficiently than the normal version of p59^{hck} (we attribute the latter to the less efficient myristoylation of p61^{hck} described above).

We conclude that it is cysteine 3 of p59^{hck} that receives the palmitate modification, that cysteine 3 can also be at least inefficiently palmitoylated when present in p61^{hck}, that myristoylation is required for the additional modification with palmitate, but that palmitoylation is not required for myristoylation (in accord with the order of addition of these modifications; see Discussion). By inspection of the amino acid sequences, we arrived at a minimal consensus sequence for dual acylation at the amino termini of proteins (Fig. 4A).

Dual acylation increases the affinity of *HCK* proteins for membranes. Having characterized the acylations of the two *HCK* proteins, we used the various alleles of *HCK* described above to explore the contribution of the acylations to subcellular localization (Fig. 6). As before, wild-type p61^{hck} was more

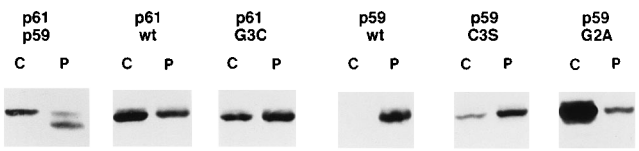

FIG. 6. Influence of myristoylation and palmitoylation on the subcellular localization of *HCK* proteins. NIH 3T3 cells expressing the wild-type (wt) and mutant *HCK* proteins were fractionated by centrifugation at 50,000 × *g* into cytosol (C) and particulate (P) fractions. Equal amounts of each fraction were analyzed by probing Western blots with a rabbit polyclonal antibody to *HCK* as described in the legend to Fig. 2. The positions of p59^{hck} and p61^{hck} are indicated.

prevalent in the cytosol than in the membrane fraction (25 to 30% of total p61^{hck} was membrane associated). However, substitution of cysteine 3 for glycine in mutant p61G3C, which results in at least inefficient palmitoylation (see above), reversed this distribution so that now 70 to 75% of p61G3C was membrane associated. Similarly, wild-type p59^{hck} was found exclusively in the membrane fraction, whereas a detectable fraction of p59C3S (which is myristoylated but not palmitoylated; see above) was in the cytosol, and the version of p59^{hck} that is neither palmitoylated nor myristoylated (p59G2A) was found almost entirely in the cytosol. We conclude that myristoylation alone provides some affinity for membranes, which is substantially enhanced by the addition of palmitate to the proteins. However, the requirement of myristoylation for palmitoylation has prevented us from assessing the effect of the latter modification in isolation.

A fraction of palmitoylated *HCK* protein is associated with caveolae. The surfaces of many cell types are covered by small invaginations known as caveolae. Recent reports have described the enrichment of several components of the signal transduction machinery in caveolae, including various glycosylphosphatidylinositol (GPI)-linked proteins and *SRC* family PTKs (8, 37, 64). Antibodies directed against a number of GPI-linked proteins have been shown to coprecipitate members of the *SRC* PTKs (9, 14, 66, 74, 75). The exact nature of the associations has not been determined, but a recent report has suggested a requirement for palmitoylation of the *SRC* PTKs (67). Thus, it seemed reasonable to ask whether *HCK* proteins might be associated with caveolae by virtue of palmitoylation.

We first examined NIH 3T3 cells expressing alleles of *HCK* from a retroviral vector as described above. Fractions of caveolae were prepared by lysis of cells with Triton X-100 followed by flotation in a continuous gradient of sucrose (Fig. 7A). In this procedure, the bulk of membrane proteins are solubilized and remain at the bottom of the gradient (40% sucrose) along with other soluble proteins; caveolae resist disruption and float to a characteristic position in a sucrose gradient (10 to 20% sucrose) (64). Caveolae were located in the gradient by analysis for the protein caveolin (Fig. 7) (15, 61) and electron microscopy (data not shown). Immunoprecipitation of caveolin cosediments a membrane population that contains p59^{hck}, suggesting that they are on the same caveolar preparations (data not shown).

Approximately 20% of the total p59^{hck} colocalized with caveolae in the gradients (Fig. 7A). The remainder behaved as soluble protein. In contrast, none of the p61^{hck} was found in association with caveolae; instead, it all behaved as soluble protein.

For the analysis of multiple samples, we found it more convenient to locate the caveolar fraction by its opacity and position in the gradient and then to remove it as a pool for analysis. The presence of caveolae in the samples was authenticated by electron microscopy (data not shown) and analysis for the protein caveolin, a prominent component of caveolae (Fig. 7B). A pool of soluble protein was also removed from the bottom of the gradient and analyzed.

As before, the caveolar fractions contained p59^{hck} but not p61^{hck}, irrespective of whether the proteins were expressed together or independently (Fig. 7B). Again, we estimated that approximately 20% of the total p59^{hck} was associated with caveolae; the remainder had been solubilized by detergent. It was also clear that palmitoylation was required for the association with caveolae: p59C3S and p59G2A were absent from the caveolar fractions, whereas a substantial fraction of p61G3C was associated with the organelles (Fig. 7B). As be-

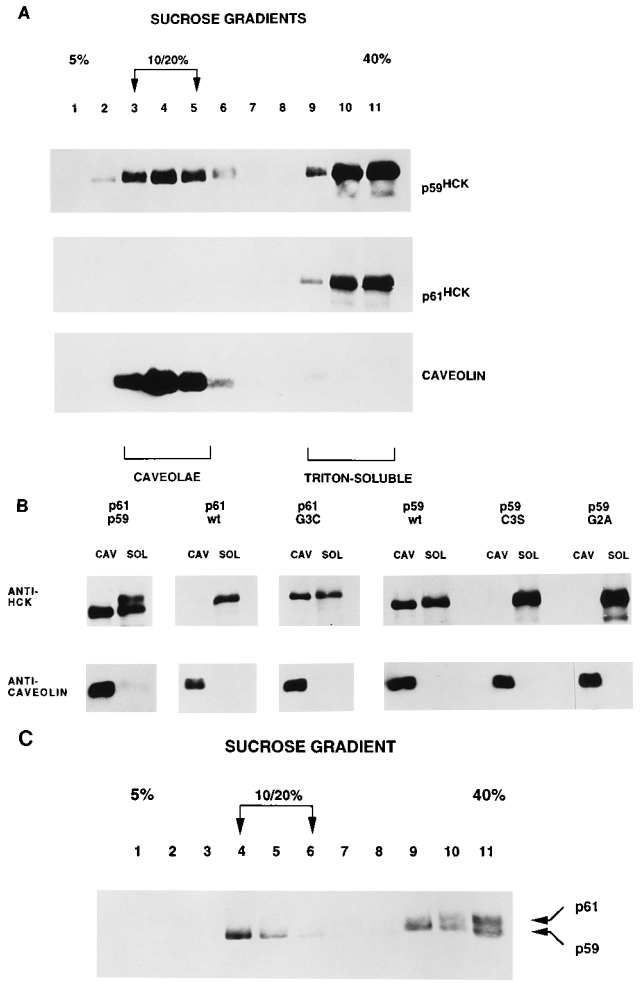

FIG. 7. Association of myristoylated and palmitoylated *HCK* proteins with caveolae. NIH 3T3 cells expressing either $p59^{hck}$ or $p61^{hck}$ (A) were lysed in 1% Triton X-100 and fractionated on linear sucrose gradients as described in Materials and Methods. Equal amounts of the 1-ml gradient fractions were analyzed by SDS-PAGE followed by Western blotting with a rabbit polyclonal to *HCK*, to detect both $p59^{hck}$ and $p61^{hck}$ (top and middle rows), and with a monoclonal antibody to caveolin (bottom row). NIH 3T3 cells expressing mutant and wild-type (wt) *HCK* proteins (B) were prepared as described above except that the caveolae (CAV) and Triton-soluble fractions (SOL) were prepared by pooling gradient fractions 2 to 5 and 9 to 11, respectively. The caveolar fraction was diluted to ca. 5% sucrose and centrifuged to pellet the caveolae; the Triton-soluble fraction was TCA precipitated. A fraction of each sample (one-fifth as much Triton-soluble fraction as caveolar fraction) was analyzed by SDS-PAGE and subsequent Western blotting with antibodies specific for HCK (top row) and caveolin (bottom row). U937 cells (C) were lysed in Triton X-100 and fractionated on a linear sucrose gradient as described for panel A. Equal amounts of the gradient fractions were analyzed by SDS-PAGE followed by Western blotting with antibodies to *HCK*.

fore, we were unable to assess the contribution of palmitoylation in the absence of myristoylation because the latter is required for the former (see above).

Fractionation of U937 cells, in which *HCK* is expressed naturally, also revealed approximately 20% of total cellular $p59^{hck}$ in the caveolar fraction of gradients (Fig. 7C). Again, no $p61^{hck}$ was detected in the caveolar fraction. Similar results were obtained with the human myelomonocytic line HL-60, with lines of mouse macrophages (RAW 264.7 and Bac1.2F5), and with U937 cells that had been differentiated with TPA (data not shown).

We also found the *SRC* family PTK encoded by *LYN* in the caveolar fractions from all of the myelomonocytic cell lines surveyed (data not shown). These findings conform to the presence of a suitable amino acid sequence at the amino termini of both isoforms of *LYN* protein (Fig. 4A) (the two isoforms have the same amino-terminal sequence [72, 84]) and to a recent report that the *LYN* proteins are associated with Thy-1 (14), a caveolar protein that is anchored to membranes by GPI (37).

We found no *SRC* protein in caveolar fractions, despite its relative abundance in several of the cell lines that we analyzed (data not shown). This conforms to previous demonstrations that *SRC* protein is neither palmitoylated nor detectable in caveolae (1, 8, 65).

Our immunological reagents failed to react with caveolin from human cells, such as U937, but we authenticated the caveolar fractions by electron microscopy, with results identical to those for NIH 3T3 cells (data not shown). In addition, we exploited the dependence of caveolar integrity upon cholesterol (62). Treatment of cells with the antibiotic nystatin, which binds and sequesters cholesterol (62), greatly reduced the amount $p59^{hck}$ found in the appropriate region of the gradients (data not shown). We conclude that ca. 20% of $p59^{hck}$ is associated with caveolae in myelomonocytic cells, in which it is expressed naturally, as well as in fibroblasts, in which it has been expressed artificially.

DISCUSSION

The two *HCK* proteins distribute differently among subcellular fractions. The two proteins encoded by *HCK* arise from a single mRNA by alternative initiations of translation (38, 57). The smaller protein ($p59^{hck}$ in human cells) is initiated at an AUG codon, whereas the larger ($p61^{hck}$ in human cells) initiates at a CUG 21 codons upstream. The larger *HCK* protein has an additional 21 amino acid residues at its amino terminus. This finding raises the possibility that $p59^{hck}$ and $p61^{hck}$ differ in properties and/or function. At present, we can point to one main difference. The two proteins differ in how they distribute among subcellular fractions (38), a major subject of this report. For the moment, however, we can place no functional significance on this finding.

From a previous report (38) and the work described here, it is clear that the two forms of *HCK* protein differ in their affinities for cellular membranes. The smaller protein is inevitably found almost entirely in crude fractions of membranes, whereas at least half of the larger protein occurs in the cytosol. Our own unpublished work indicates that removal of *HCK* proteins from membranes requires either alkali or nonionic detergent, indicating that they are tightly bound peripheral membrane proteins and in this regard mimic the *SRC* protein (25). Until now, however, the nature of the membranes with which *HCK* proteins associate had not been explored in any detail.

Here we have shown that ca. 20% of $p59^{hck}$ in either human myelomonocytic cells or mouse fibroblasts is associated with caveolae, approximating recent results with the *LCK* and *FYN* proteins (59, 65). We acknowledge that the conclusion must be considered provisional. However, for the conclusion to be wrong, cells would have to contain an additional membranous organelle with all of the following properties: resistance to disruption by Triton X-100, a buoyant density in isopynic sucrose gradients identical to that of caveolae, the presence of caveolin (in fibroblast cells), and dependence upon cholesterol for integrity. In contrast, $p61^{hck}$ appears not to associate with caveolae. This difference provides the first substantial indication that the functions of the two *HCK* proteins may differ.

We do not know the topographical orientation of p59^{hck} on caveolae, but we presume that the protein is attached to the cytoplasmic surface of the organelles and that the catalytic domain of the protein is exposed to the cytoplasm. These presumptions are based on previous studies of how other members of the *SRC* family of PTKs associate with cellular membranes (53), although most of these studies have been performed with the *SRC* protein itself, which does not associate with caveolae (this study and references 8 and 65), and there has been no report of work addressing the topography of *SRC* family PTKs on caveolae.

The remainder of p59^{hck} is also firmly bound to membranes, but the nature of these membranes is not known. In the fractionations reported here, all membranous organelles other than caveolae have been solubilized with detergent. Approximately 50% of p61^{hck} is also bound to membranes of unknown sorts, which were solubilized in our fractionations. Thus, it remains to be seen whether any of the p59^{hck} and any of the p61^{hck} share locales within the cell.

Modification of *HCK* proteins by both myristoylation and palmitoylation. All of the known PTKs in the *SRC* family are myristoylated on their amino termini (53, 54). In addition, it is now clear that all but three of the proteins are palmitoylated (Fig. 4A). The myristoylation occurs on a glycine at the amino terminus, exposed by prior removal of the initiating methionine residue (20). Palmitoylation occurs on a cysteine at residue 3 in the proteins and/or on a cysteine at residue 5, when such is present (1, 29, 44, 59, 65, 67). The *SRC* family PTKs share this dual acylation with several of the G_α signalling proteins (22, 33, 48, 78) and perhaps other peripheral membrane proteins as well. Myristoylation is a cotranslational event (20), whereas palmitoylation occurs subsequent to the completion of translation (1, 44).

A survey of pertinent amino acid sequences revealed what we propose as the minimum consensus for dual acylation at the amino terminus of proteins: Met-Gly-Cys-X-X-Ser/Cys-X* (where the asterisk denotes some specificity for certain amino acids; see reference 24, 76, and 77 and Fig. 4A). Within this consensus, the character of residues 2 and 7, at least, is important to myristoylation (20, 24, 76, 77), but the determinants of palmitoylation (beyond the necessary cysteine recipient) have not been explored.

For want of a suitable cysteine residue, p61^{hck} is not palmitoylated. In addition, an appreciable fraction of this protein may not be myristoylated, although the evidence for this is not decisive. We note that the seventh amino acid in p61^{hck} may not be favorable for myristoylation (20, 24). By contrast, it seems likely that virtually all of p59^{hck} is myristoylated, because the protein is found exclusively in membrane fractions (this distribution cannot be attributed to palmitoylation alone because the modification requires myristoylation). If this surmise is correct, we can in turn conclude that both forms of acylation are present on single molecules of p59^{hck}. The requirement of myristoylation for palmitoylation in itself suggests the same conclusion: it is difficult to see how such a requirement could operate in *trans.*

The role of lipid modifications in the binding of *HCK* proteins to membranous organelles. Among the *SRC* family PTKs, myristoylation had been commonly found as a requirement for attachment to membranes (53, 54). Thus, it came as no surprise to learn that a mutant version of p59^{hck} that cannot be myristoylated is located almost entirely in the cytosol. It is widely assumed that the myristate moiety facilitates binding to membranes by hydrophobic insertion into the lipid bilayer (53, 54), but there is also evidence that other factors, including electrostatic interactions between protein domains and membrane lipids (6, 68) and protein-protein interactions (18, 56), contribute to binding.

A portion of p61^{hck} is found in the cytosol, a finding that was attributed previously to the absence of myristate (38). But we have now found that at least some of the cytosolic p61^{hck} is, in fact, myristoylated, and so this modification in itself is not always sufficient for stable anchorage of a protein to membranes. The point is dramatized by the increase in affinity for membranes displayed by p61^{hck} that is both myristoylated and palmitoylated. Similarly, we surmise that dual acylation contributes to the complete association of p59^{hck} with membranes.

It appears that myristoylation is required for palmitoylation of p59^{hck}. We imagine that this requirement may reflect the following sequence of events: cotranslational myristoylation of p59^{hck}, attachment of the nascent or completed protein to membranes, consequent accessibility to the machinery for palmitoylation, and eventual posttranslational palmitoylation. Thus, the requirement of myristoylation for palmitoylation might represent the need for membrane attachment before the latter modification can occur. In this scheme, palmitoylation could not be a requirement for myristoylation, and that is in fact the case both for p59^{hck} (this study) and other proteins (1, 22, 29, 43, 59, 67). On the other hand, this scheme cannot be universal because some of the G proteins (e.g., $G_{\alpha s}$ and $G_{\alpha q}$) are palmitoylated and bind to membranes in the absence of myristoylation (Fig. 4A and references 13 and 81).

What is the role of palmitoylation in the function of p59^{hck}? Two answers are presently apparent. First, addition of palmitate to a myristoylated *HCK* protein increases the affinity of that protein for membranes. Hence, p59^{hck} is more securely attached to cellular membranes than is p61^{hck}, and p61^{hck} that is palmitoylated as a consequence of mutation has a greater affinity for membranes than does the unpalmitoylated wild-type protein. Second, it appears that palmitoylation is required for association of p59^{hck} with caveolae, as is the case for the *FYN* PTK (65). Moreover, palmitoylation of the mutant p61G3C allows a portion of the protein to associate with caveolae, and palmitoylation alone appears to facilitate association of $G_{\alpha s}$ with those organelles (8, 58). Thus, palmitoylation alone may in some instances suffice for association of proteins with caveolae. We were unable to assess this possibility for p59^{hck} because antecedent myristoylation is required for palmitoylation.

Since it is readily reversible by enzymatic machinery within the cell (7), palmitoylation may also represent a means by which the function of p59^{hck} can be regulated. There is precedent for this thought in the recent finding that signalling through a hormone receptor leads to rapid depalmitoylation of a G_α protein and consequent translocation of the protein into the cytoplasm (80). Similarly, depalmitoylation would provide a means by which to rapidly dissociate p59^{hck} from caveolae. In addition, only 20% of palmitoylated p59^{hck} is associated with caveolae at any given time. Thus, the modification may serve to target p59^{hck} to other membranous organelles as well.

The role of *HCK* proteins in signalling. A number of the *SRC* family PTKs have been implicated in cellular signalling (63). In many instances, the PTK serves as a signal transducer for a transmembrane protein that does not itself possess an enzymatic activity. Perhaps the best-explored examples come from lymphocytes, in which several *SRC* family PTKs serve to transmit signals from transmembrane proteins to targets within the cells (82). This signalling appears to serve vital roles in the development and activation of lymphocytes (50). Similarly, there are clues that the PTK encoded by *HCK* may help engender the intracellular response to a variety of extracellular agents (16, 17, 19, 35, 73, 79).

The finding that p59^{hck} is associated with caveolae may help refine studies on the role of this protein in cell signalling. Caveolae were first encountered in electron micrographs, as vesicular invaginations of the plasma membrane known also as plasmalemmal vesicles (45–47, 83). Early studies found caveolae in polarized endothelial cells, leading to the suggestion that these organelles might mediate transcytosis (69–71). More recently, however, caveolae have been found in diverse other cells, and suggestions regarding their possible function now also include potocytosis (4) and sites for signal transduction (2, 3, 36).

The clustering of signalling elements in caveolae may allow for functional overlap among these elements. For example, engagement of the receptor for LPS activates *HCK* kinase in myelomonocytic cells (16, 73), yet mice that are homozygous for a null mutation of *HCK* display no defect in LPS signalling (39). The closely related PTK encoded by *LYN* is also present in caveolae and thus might complement deficiencies in *HCK*. Indeed, the amounts of *LYN* protein and kinase activity are increased in macrophages derived from the mutant mice, suggesting a compensatory response (39, 57).

A prominent feature of caveolae is the clustering there of diverse proteins that are anchored to the cell surface by GPI (5, 9, 37, 85, 87). At least some of these proteins presumably signal to the interior of the cell by means of an associated transmembrane protein, which in turn activates an intracellular signalling device (14, 66, 73–75). We anticipate that p59^{hck} is one such device within the signalling machinery associated with caveolae. Thus, a focus on the components of caveolae may be a useful strategy in the search for proteins that lie either upstream or downstream of p59^{hck} in signalling pathways.

ACKNOWLEDGMENTS

We thank Ursula Lichtenberg for the production of the *HCK* antisera and Mai Lie Wong for help with the electron microscopy. We thank Sherrie Hans and Harold Varmus for their advice and helpful discussions during the course of the work.

This work was supported by the National Institutes of Health (grant CA 44338) and by funds from the G. W. Hooper Research Foundation. S.M.R. was supported by a postdoctoral fellowship from the Human Frontiers Science Program Organization and most recently by senior postdoctoral fellowship S-15-94 from the American Cancer Society, California Division.

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