Uridine Phosphorylase Association with Vimentin

INTRACELLULAR DISTRIBUTION AND LOCALIZATION*

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Uridine phosphorylase (UPase), a key enzyme in the pyrimidine salvage pathway, is associated with the intermediate filament protein vimentin, in NIH 3T3 fibroblasts and colon 26 cells. Affinity chromatography was utilized to purify UPase from colon 26 and NIH 3T3 cells using the uridine phosphorylase inhibitor 5′-amino benzylacyclouridine linked to an agarose matrix. Vimentin copurification with UPase was confirmed using both Western blot analysis and MALDI-MS methods. Separation of cytosolic proteins using gel filtration chromatography yields a high molecular weight complex containing UPase and vimentin. Purified recombinant UPase and recombinant vimentin were shown to bind in vitro with an affinity of 120 pm and a stoichiometry of 1:2. Immunofluorescence techniques confirm that UPase is associated with vimentin in both NIH 3T3 and colon 26 cells and that depolymerization of the microtubule system using nocodazole results in UPase remaining associated with the collapsed intermediate filament, vimentin. Our data demonstrate that UPase is associated with both the soluble and insoluble pools of vimentin. Approximately 60–70% of the total UPase exists in the cytosol as a soluble protein. Sequential extraction of NIH 3T3 or colon 26 cells liberates an additional 30–40% UPase activity associated with a detergent extractable fraction. All pools of UPase have been shown to possess enzymatic activity. We demonstrate for the first time that UPase is associated with vimentin and the existence of an enzymatically active cytoskeleton-associated UPase.

The ability of cells to maintain a constant supply of pyrimidine and purine nucleotides is dependent on both de novo synthetic and salvage pathways. The relative importance of either the de novo or the salvage pathway in the maintenance of nucleotide pools is variable and dependent on the cell or tissue type (reviewed in Refs. 1, 2). Uridine phosphorylase (UPase) is an important enzyme in the pyrimidine salvage pathway and catalyzes the reversible phosphorylation of uridine to uracil (3–5). This enzyme is present in most human cells and tissues analyzed, and it is frequently elevated in tumors (4, 5).

Enzymatic activity may also be induced in different cell lines by cytokines such as tumor necrosis factor-α, interleukin-1α, and interferon-α and -γ as well as vitamin D3 (6–8). UPase has also been shown to be important in the activation and catabolism of fluoropyrimidines (9, 10), and the modulation of its enzymatic activity may affect the therapeutic efficacy of these chemotherapeutic agents (11, 14).

UPase also plays an important role in the homeostatic regulation of both intracellular and plasma uridine concentrations (11–14). Uridine plasma concentration is under very stringent regulation (15, 16) mostly as a function of liver metabolic control (17), intracellular UPase enzymatic activity (11–14), and cellular transport by both facilitated diffusion and Na+-dependent active transport mechanisms (18–25). Uridine is critical in the synthesis of RNA and biological membranes through the formation of pyrimidine-lipid and pyrimidine-sugar conjugates (reviewed in Ref. 1), and it has been associated with the regulation of a number of biological processes (1).

Whereas there is evidence that uridine and its nucleotides are associated with different biological processes (reviewed in Refs. 1 and 2) the precise mechanisms that allow uridine to modulate these processes are not well defined. Uridine has been shown to cause increased vascular resistance (25), hyperpolarize amphibian and rat ganglia (26, 27), potentiate dopaminergic transmission, and reduce anxiety in animal models (28, 29), as well as potentiate barbiturate effects and induce sleep in rats (30, 31). Uridine perfusion has been shown to maintain brain metabolism during ischemia (32, 33) and to rapidly restore myocardial ATP and UDPG (34) following myocardial ischemia.

Characterization of UPase intracellular localization and association with other proteins may provide some insight into the mechanisms that control uridine metabolism in cells. In this study, we characterize the cellular distribution and the associated enzymatic activity of UPase.

EXPERIMENTAL PROCEDURES

Materials—Primary antibodies against UPase were prepared at Yale (rabbit anti-UPase polyclonal antibody to human recombinant UPase) (35), or purchased from Sigma Chemical Co. (St. Louis, MO) (mouse anti-Vimentin Clone V9). Secondary antibodies, horseradish peroxidase-conjugated donkey anti-mouse or anti-rabbit (fluorescein isothiocyanate-conjugated and Texas red-conjugated donkey anti-rabbit) or (fluorescein isothiocyanate- or Texas red-conjugated sheep anti-mouse) were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Antibodies for vimentin (V9 or monoclonal antibody clone 13.2) or polyclonal goat anti-vimentin were purchased from Sigma (St. Louis, MO). Immunoaffinity support for antibody immobilization and antibody purification was purchased from Pierce. Affi-Gel-10-activated agarose and protein assay reagent were purchased from Bio-Rad (Hercules, CA). 5′-Amino benzylacyclouridine and benzylacyclouridine (BAU) were
A generous gift from Dr. S. Chu at Brown University (Providence, RI). 

Tissue Culture—Cells (NH 3T3 and colon 26) were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Sigma) and were maintained in a humidified atmosphere containing 5% CO2 in air. NIH 3T3 cells were purchased from ATCC, and colon 26 cells were derived from the solid tumor. Colon 26 cells were grown in tissue culture standard procedures. Colon 26 cells were originally obtained from Southern Research Institute (Birmingham, AL) and grown subconfluently in BALB/C mice.

Affinity Chromatography—The BAU affinity column was prepared by coupling amino-BAU to an Affi-Gel-10-agarose matrix as previously described (35). Affinity chromatography columns were prepared using a cyanoborohydride coupling procedure (Pierce) following manufacturer guidelines. Vimentin antibody was purchased from Sigma (goat anti-vimentin and clone V9 mouse anti-vimentin were both used to prepare affinity columns).

In all affinity chromatographic procedures, cells (colon 26 or NIH 3T3) or solid tumors (colon 26) were lysed in 50 mM Tris-HCl, pH 7.4 (containing 2 mM dithiothreitol), using a Dounce homogenization apparatus or tissue homogenizer, respectively. Cell lysates were prepared at 4 °C, and the supernatant from the 30,000 × g centrifugation was applied to the column. Following sample application, the column was washed with ~10 volumes of buffer or until the column effluent contained no detectable protein (Bio-Rad Coomassie Brilliant Blue G-250 dye reagent). Sample elution was accomplished using 4–8 column volumes of 0.1 M glycine (affinity column affinity) or 20 mM uridine (BAU affinity column). The eluted proteins were concentrated using Ultrafree-4 centrifugal filters (Millipore) and analyzed by SDS-polyacrylamide gel electrophoresis and Western blot techniques.

Gel Filtration Chromatography—Cell lysates from colon 26 cells grown as a monolayer in 150-cm2 dishes were prepared in 20 mM Tris-HCl, 137 mM NaCl, pH 7.4 (TBS). Cytosolic fractions were centrifuged at 100,000 × g for 1 h and applied to a Sephacryl S-300 column (Amersham Pharmacia Biotech) that had been calibrated using known molecular weight markers (Amersham Pharmacia Biotech). The mobile phase was 20 mM Tris-HCl, 137 mM NaCl with a flow rate of 1 ml/min. Protein elution of standards was monitored in fractions using the Bio-Rad Coomassie Brilliant Blue G-250 dye reagent.

Immunofluorescence Techniques—For whole cell immunofluorescence analyses, cells were grown to 50–70% confluence on glass cell culture slides. After a brief wash with PBS, cell monolayers were fixed with 3.8% paraformaldehyde in PBS for 10 min at 4 °C. Fixed cells were washed briefly in PBS (5 min) and permeabilized using 0.1% Triton X-100 in PBS (10 min), and nonspecific binding was blocked using 3% BSA or serum of the same specificity as the secondary antibody when available (10 min). Incubation with the primary antibody was performed for 1 h at room temperature. After washing the excess unbound antibody (two times 5 min with PBS), sample was exposed for 1 h to secondary antibody at room temperature. The excess secondary antibody was washed with PBS, and the slides were mounted in fluorescent antibody-compatible medium (Molecular Probes, Eugene, OR). Photographs were taken using a Zeiss Axioshot microscope and camera apparatus. All experiments included a negative nonspecific serum (preimmune) control to ensure specificity of the observed fluorescence. In the case of dual antibody detection, reagent compatibility was determined using normal or preimmune serum and secondary antibodies as negative controls singly and in combination.

Cytoskeleton immunofluorescence was performed as described previously (36). Cells grown to 70% confluence on glass slide covers were rinsed briefly in PBS, and then soluble proteins were extracted for 3–5 min at room temperature using cytoskeleton buffer (100 mM PIPES, pH 6.9, 1 mM MgCl2, 1 mM EGTA containing 4% polyethylene glycol and 1% Triton X-100). Following extraction, cell cytoskeletons were rinsed three times with cytoskeleton stabilizing buffer without detergent. The immunofluorescence analysis was performed as described above.

MALDI-MS—The unknown protein band was excised from a Coo massie Blue-stained gel and digested overnight using trypsin as described (37). The peptides were subsequently analyzed using MALDI-MS and the Profound peptide data base at the Howard Hughes Medical Institute Biopolymer/W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University (38, 39).

RESULTS

Uridine phosphorolase was purified from NIH 3T3 and colon 26 cells using an affinity column coupled to the high affinity uridine phosphorolase inhibitor, 5′-amino-benzylacyclouridine. For colon 26 cells, solid tumor homogenate was affixed to the BAU column. The Coo massie Blue-stained blot of UPase eluted using 20 mM uridine is shown in Fig. 1 (lane 1). The one-step purification procedure results in the isolation of UPase and the
copurification of a 58-kDa species. The identification of the lower band as UPase using Western blot analysis is shown in Fig. 1 (lane 2). No cross reactivity of the UPase antibody with the upper band was noted. The lower band at 34 kDa in Fig. 1, lane 1, represents purified UPase, and the upper band (~58 kDa) was identified as vimentin using MALDI-MS of the tryptic digest (Fig. 2). The tryptic digest of the Coomassie Blue-stained 58-kDa band excised from acrylamide gel covered 57% of the protein using a mass tolerance of ±0.2 atomic mass unit for monoisotopic and ±0.5 atomic mass unit for observed average masses.

We analyzed NIH 3T3 cells to evaluate whether the copurification of UPase with vimentin was a phenomenon specific to colon 26 tumor cells, which contain highly elevated levels of UPase. UPase was purified from NIH 3T3 and colon 26 cell lines using the BAU column as described, and the results of the Western blots are shown in Fig. 3 (A and B), respectively. In both cases, vimentin was identified in the BAU eluate using clone V9 monoclonal antibody as shown in Figs. 3A, lane 2, and 3B, lane 2.

We, and others (35, 40) have previously shown that the majority of the UPase in cells exists in a soluble form that is readily extractable using near-physiologic buffers (40). This is in contrast to what is known about the intermediate filament vimentin, which is one of the most insoluble proteins known. In fact, it has been shown that less than 1% of the total vimentin exists in cells as soluble tetramers (41). The fact that UPase and vimentin are copurified in cell/tumor extracts using physiologic buffers suggests that a fraction of UPase exists in combination with this soluble pool of vimentin. To confirm the association of UPase with vimentin in the cytosol, we performed gel filtration chromatography using a Sephacryl-s300 column to separate the UPase monomer from that which is associated with vimentin. As shown in Fig. 4A, UPase elutes from the column as two distinct peaks. The high molecular mass species elutes at ~400–500 kDa, and the low molecular mass peak elutes as a broad peak with a mean value of about 34 kDa, suggesting that it exists predominantly as a monomer in the cytoplasm. The shoulder on the low molecular mass peak may indicate that UPase exists in both monomeric and dimeric forms or is associated with another protein, under these experimental conditions. Vimentin elutes from the Sephacryl-s300 column in the same fractions containing the high molecular mass UPase, again suggesting they exist in the cytosolic pool as a complex. The Western blot shown in Fig. 4B shows the UPase and vimentin present in each fraction depicted in Fig. 4A.

Confirmation of the association of UPase with the soluble pool of vimentin was also confirmed using a combination of gel filtration chromatography and immune precipitation (data not shown).

The stoichiometry of the UPase-vimentin complex, was estimated using a slot blot binding assay to measure direct protein-protein interactions. Purified recombinant human UPase was directly coupled to horseradish peroxidase and used to probe known concentrations of purified recombinant vimentin (inset, Fig. 3). UPase was purified from NIH 3T3 (A) and colon 26 cells (B) using a BAU affinity column. Lane 1 (A and B) are the Western blots of the UPase isolated from the affinity column for NIH 3T3 and colon 26, respectively. Lane 2 (A and B) are the same Western blots re-analyzed for vimentin using clone V9 monoclonal antibody as described.

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Concentrations of 25, 12.5, 6.25, 3.2, 1.7, and 0.85 mM Tris were affinity-purified using anti-vimentin chromatography. The detergent extracts were utilized to solubilize cytoskeleton (including vimentin) and associated proteins while maintaining lower antibody efficiency in the presence of detergent. The stoichiometry of UPase binding to vimentin is calculated at ~1:2 and the $K_d$ of the complex is 120 pm.

We determined whether UPase could be found in combination with the polymeric form of vimentin, so NIH 3T3 cells and colon 26 cells were sequentially extracted using Tris-buffered saline, followed by 1% Triton X-100, and finally RIPA buffer. The detergent extracts were utilized to solubilize cytoskeleton (including vimentin) and associated proteins while maintaining optimum enzyme activity (Table I) and UPase-vimentin association (Fig. 6). Extracts from each of these conditions were affinity-purified using anti-vimentin chromatography. The results from these experiments demonstrate that UPase and vimentin exist together in a complex in each of these fractions. Fig. 6B shows a Western blot of UPase isolated from colon 26 cell lysates purified using anti-vimentin affinity column and indicates the presence of a UPase-vimentin complex in each of these fractions. Although the extraction of vimentin from both the Tris and Triton X-100 fractions are similar (Fig. 6A, lanes 1 and 2), less vimentin was retained on the affinity column in the RIPA solubilized fraction, probably a function of lower antibody efficiency in the presence of detergents contained in the RIPA buffer. The predominant form of UPase exists in the Tris-soluble fraction in combination with the soluble tetrameric vimentin. Further solubilization of the cell pellet with Triton X-100 liberates a smaller percentage of UPase (~15–30% based on enzymatic activity shown in Table I) suggesting less UPase is associated with the polymeric membrane-associated form of vimentin liberated using this technique. Finally, there is a very small percentage of UPase released by the final RIPA solubilization of the pellet, which is between 10–20% based on enzymatic activity (Table I) and is difficult to visualize on the Western blot shown in Fig. 6. This additional pool of UPase is also associated with the polymeric form of vimentin. Taken together, both the Triton X-100 and RIPA buffer extracted UPase represent a pool of 25–50% additional UPase enzymatic activity found in association with the polymeric and membrane-associated vimentin pool.

Western blots of NIH 3T3 (lanes 1–3) and colon 26 cells (lanes 4–6) sequentially extracted without detergent (lanes 1 and 4), using 1% Triton X-100 (lanes 2 and 5) and RIPA buffer (lanes 3 and 6) are shown in Fig. 7. The distribution pattern of vimentin in these fractions is shown in the upper panel (A), and the distribution of UPase in the same fractions is shown in the lower panel (B). Although UPase and vimentin copurify in all three fractions as determined by affinity chromatography (Fig. 6), there is an inverse relationship between the relative abundance of each protein in these fractions. Although the majority of UPase exists in the Tris-buffer-soluble fraction (lanes 1 and 4) for NIH 3T3 and colon 26 cells, respectively, less than 1% of the total vimentin has been shown to exist in this pool (41) (Fig. 7A, lanes 1 and 4). Although 99% of the vimentin exists in the polymeric form and is extractable using detergents (Fig. 7A, lanes 3 and 6), less than 20% of the total UPase is present in this fraction (RIPA) based on enzyme activity (Table I).

Further evidence for the association of UPase with the polymeric form of vimentin was demonstrated using NIH 3T3 cells from which soluble proteins were extracted in the presence of cytoskeleton stabilizing agents (36). We extracted NIH 3T3 cells grown on glass slides with cytoskeleton-stabilizing buffer containing 1% Triton X-100 and 4% polyethylene glycol as described (36) before fixing and processing them for immunofluorescent microscopy. Subcellular localization of UPase in NIH 3T3 demonstrated a distinctly filamentous pattern (Fig. 8A). The staining for UPase was particularly intense in the perinuclear area of the cell and extends outward toward the cell periphery within a filamentous network, which is identical to the intermediate filament vimentin as shown in Fig. 8B. Regions of the cells where vimentin and UPase colocalize are shown in Fig. 8C and appear yellow.

Because the intermediate filament network is found in close vicinity, it appears that UPase and vimentin are present in the same complex, similar to that demonstrated for other enzymes (42). The localization of UPase in the cell periphery suggests a role in transport of vimentin to the filamentous network. This transport mechanism is consistent with that demonstrated for other proteins, such as tubulin (43) and actin (44), which are transported through the intermediate filament network from the cell periphery to the perinuclear region. This observation is consistent with the localization of UPase to the intermediate filament network in NIH 3T3 cells, which demonstrates a distinctly filamentous pattern. The staining for UPase is particularly intense in the perinuclear area of the cell and extends outward toward the cell periphery within a filamentous network, which is identical to the intermediate filament vimentin as shown in Fig. 8B. Regions of the cells where vimentin and UPase colocalize are shown in Fig. 8C and appear yellow.

**Table I**

| Cell line | Tris | Triton X-100 | RIPA |
|-----------|------|-------------|------|
| NIH 3T3   | 62.1 (6.0) | 22.7 (8.2) | 15.3 (2.3) |
| Colon 26  | 59.3 (2.9) | 24.8 (4.1) | 15.8 (4.9) |
We have shown that UPase and the intermediate filament protein vimentin are colocalized using immunofluorescent antibody techniques, affinity chromatography, gel filtration, and immunoprecipitation. We have further shown these proteins interact in vitro using binding assays. The difficulty is in determining the physiological relevance of this observation. The role of uridine phosphorylase in the salvage pathway of pyrimidine nucleoside biosynthesis does not readily translate into a role for this enzyme in association with the cytoskeleton. Additional difficulty in interpreting these data lies with the inability to clearly establish a functional role for the intermediate filament vimentin. Although a number of theories have been proposed for the function of this network, the data are not conclusive. Cellular processes as diverse as differentiation, motility, signal transduction, cell division, cytoskeletal stability, and vesicular trafficking have been associated with alterations in the dynamics of the intermediate filaments (reviews in Refs. 44–48, and references within). Deletion of the vimentin protein in mice had no detrimental characteristics, and the mice apparently developed and reproduced normally (49). It has recently been shown that vimentin null mice exhibit neurological defects and impaired motor coordination (50). Further in vitro analyses of fibroblasts isolated from wild type and vimentin null mouse embryos show that vimentin null cells exhibit reduced mechanical stability, decreased growth factor-directed and random motility, and reduced capacity to cause contraction of collagen fibrils (51), a process necessary in wound healing.

In recent years, a number of proteins have been shown to be associated with the vimentin intermediate filament scaffold, including p53 (52), protein kinase C (53), Yes and cGMP kinase (54, 55), glycolytic enzymes pyruvate kinase, creatine kinase, and glyceraldehyde-3-phosphate dehydrogenase (56–58), and nucleoside diphosphate kinase (NDPK) (56, 59) as well as the cross-linking proteins plectin, IFAP-300, and filamin, which link intermediate filaments to other cytoskeletal elements and membranes (59–63). It is particularly interesting to note the number of proteins involved in signal transduction and energy metabolism that have been associated with vimentin. Although the phenotype of the vimentin knockout mice is not evident under “normal” conditions, the recent observations of reduced mechanical strength and the cellular response to motility stimulating growth factors in fibroblasts isolated from these animals supports a role for the vimentin three-dimensional network in the coordination of these responses.

The proposed role for nucleoside diphosphate kinase (NDPK, nm23) in nucleotide channeling (59), production of most cellular non-ATP nucleoside triphosphates (64), and copurification with vimentin and enzymes involved in ATP formation/regeneration (56), together with our observation of UPase colocalization with this same cellular machinery, suggests that such observations are biologically relevant. If vimentin serves a largely structural role in cellular homeostasis, the localization of the vimentin-associated proteins within the milieu of the cell may represent a mechanism for “docking” these proteins to the cytoskeleton scaffold. In this case, proteins associated with glycolytic processes and signal transduction may be bound to vimentin as a mechanism of sequestration of enzymatic activity or signal transduction. If the vimentin network serves as a functional scaffold that directs mRNA and vesicular trafficking, as proposed (reviewed in Ref. 45), the association of glycolytic, UPase, NDPK, and signal transduction molecules with this filamentous network may be under dynamic control. In the case of UPase, it may be relevant that a large fraction of this enzyme is associated with the soluble pool of vimentin. Because soluble vimentin represents the fraction of this protein that is added to existing filaments in response to changing cell dynamics, it seems relevant that this form of the filament is associated with UPase. Particularly, if newly synthesized vimentin targets areas of mRNA translation, having a pyrimidine degradation enzyme in close proximity to areas of high mRNA translation would seem reasonable.

It is possible that the UPase complex with vimentin represents the biologically active form of this enzyme. It has been shown by Vita et al. (65) that in Escherichia coli B., enzymatically active UPase exists as a tetramer. From our observations in colon 26 cells, the majority of soluble UPase (55–60%) exists as a monomer and the remaining UPase is found in association with the soluble vimentin tetramer, possibly in a 1:2 stoichiometry as suggested by in vitro binding assays. It is possible...
that the biologically active species of UPase is the UPase-vimentin multimer. Alternatively, it is possible that in mammalian cells UPase could exist predominantly either as a monomer or an easily dissociated tetramer not detected with our techniques.

The association of proteins with the cytoskeleton may serve different functions, including activation or inactivation (reviewed in Ref. 66) of enzymatic activity, localization of a particular enzymatic activity to multiprotein complexes (67–69), or sequestration of proteins from the soluble or nuclear pool (52, 69). Whether any or all these possibilities are true for UPase sequestered to the vimentin filaments remains to be proven. In vitro enzymatic analyses of the detergent-extractable pool of UPase demonstrated that this source of enzyme retains enzymatic activity. It is difficult to say whether this is true in the intact cell where it exists in an insoluble form. The UPase found in association with the polymeric vimentin network may represent a pool of enzyme able to mobilize that the biologically active species of UPase is the UPase-vimentin multimer. Alternatively, it is possible that in mammalian cells UPase could exist predominantly either as a monomer or an easily dissociated tetramer not detected with our techniques.

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