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Copurification of Vimentin, Energy Metabolism Enzymes, and a MER5 Homolog with Nucleoside Diphosphate Kinase

IDENTIFICATION OF TISSUE-SPECIFIC INTERACTIONS*

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Chromatography on immobilized antibodies specific to nucleoside diphosphate (NDP) kinase was utilized for affinity purification of this enzyme from detergent extracts of frog heart post-mitochondrial fractions. SDS-polyacrylamide gel electrophoresis analysis of eluates from these supports shows that five polypeptides co-purify with nucleoside diphosphate (NDP) kinase. Tryptic digests of each band were analyzed by mass spectrometric microsequencing. Data base searches by peptide mass matching and sequence homology led to the identification of these proteins as glyceraldehyde-3-phosphate dehydrogenase (40 kDa), creatine kinase (45 kDa), vimentin (55 kDa), pyruvate kinase (60 kDa), and a putative member of the antioxidant protein family (28 kDa). Distinct protein compositions were found in eluates of lung and liver extracts processed in a like manner. The 28-kDa band and vimentin were associated with NDP kinase from all tissues, but co-purification of pyruvate kinase was seen only in liver, while creatine kinase and glyceraldehyde-3-phosphate dehydrogenase were absent from eluates from lung and liver. The results suggest that while NDP kinase is associated with vimentin intermediate filaments and an antioxidant protein in most tissues, it interacts with energy metabolism enzymes in a tissue-specific manner.

In recent years NDP kinase[1] has been found to have unexpected roles, in addition to its well known ability to catalyze transfer of phosphate groups from trinucleotides to dinucleotides (1). NDP kinases are involved in growth, differentiation, development, tumor progression, metastasis, and apoptosis (reviewed in Ref. 2). NDP kinase participates in muscarinic K^+ channel opening (3), is a transcription factor for c-*myc* (4) and a protein kinase as well (5, 6). These varied functions seem to be accomplished by a limited number of gene products: to date, complete cDNAs encoding 4 human NDP kinases (nm23-H1, nm23-H2, DR-nm23, and nm23-H4) have been identified (7–9). Also, in *Dictyostelium* a separate nuclear gene encodes a mitochondrial NDP kinase (10). Although other NDP kinase genes may be identified in the future, it is unlikely that each of the multiple functions associated with this protein is performed by a distinct isoform. Differential distribution of NDP kinase isoforms (11) may account for adaptation to the requirements of specific cell types. Additionally, interaction of distinct isoforms with cell-specific factors, conceivably other proteins, could govern NDP kinase function in different tissues. This in turn might explain apparently contradictory findings regarding the connection between NDP kinase and cancer (2): while in some cell types, studies of NDP kinase expression suggest that it has a metastasis suppressor function, in other systems this relation between expression of NDP kinase and metastatic potential is either nonexistent or operates in the opposite direction.

The objective of the present work was to identify proteins that interact with NDP kinase in cardiac muscle. Immobilized antibodies to frog NDP kinase were utilized for affinity purification of proteins from frog heart extracts, and the most abundant polypeptides co-purifying with NDP kinase were identified through microsequencing. The results indicate that in heart, NDP kinase is associated with energy metabolism enzymes, an intermediate filament protein, and a 28-kDa protein homologous to antioxidant proteins. Application of the same approach to lung and liver extracts indicates that not all of these proteins interact with NDP kinase in tissues where they are expressed. In particular, copurification of energy metabolism enzymes with NDP kinase appears to be a tissue-specific phenomenon.

EXPERIMENTAL PROCEDURES

Materials

The monoclonal antibody to desmin (DE-U-10), E-64 (*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane), and rabbit muscle creatine kinase (CK), pyruvate kinase (PK), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from Sigma. Triton X-100 (Surfact Amps grade) and dimethylpimelimidate were from Pierce. Sodium deoxycholate (Ultrol grade) was from Calbiochem. Goat anti-human vimentin, goat anti-rabbit muscle PK, goat anti-CK (MM), and monoclonal antibody to GAPDH were from Chemicon. Other sources were as described (12).

Methods

Purification of frog skeletal muscle NDP kinase and specific polyclonal antibodies, as well as immunoprecipitation, SDS-PAGE and immunoblotting were as described (12). The antibodies (B2) chosen for these experiments have a high immunoprecipitation titer and show low potency for inhibition of NDP kinase activity. Neutralization of activity is sometimes associated with disruption of protein-protein complexes; thus, these antibodies are less likely to interfere with associations between NDP kinase and other proteins. IgG fractions were isolated on protein A columns (Pierce). Antibodies specific to NDP kinase were obtained by chromatography of protein A-purified IgG on NDP kinase immobilized on NHS-HiTrap cartridges (Pharmacia) or Amino-Link columns (Pierce), using protocols suggested by the manufacturers. To prepare immunoaffinity supports, 7.5 mg of total purified IgG or 1.2 mg

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[1] The abbreviations used are: NDP kinase, nucleoside diphosphate kinase; IF, intermediate filament; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CK, creatine kinase; PK, pyruvate kinase; E-64, (trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane); DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; MOPS, 4-morpholinepropanesulfonic acid.

of affinity purified IgG were cross-linked to 1 ml of protein A-Sepharose with dimethylpimelimidate (13).

Immunoaffinity Purification—Bullfrog (*Rana catesbeiana*) hearts were removed, perfused with Ringers solution to remove blood, and frozen in liquid nitrogen. Lungs and livers were dissected and frozen in liquid nitrogen. All manipulations were performed at 4 °C. Frozen tissue (0.4–0.6 g) was homogenized in 5 ml of TNE buffer (20 mM Tris·HCl, pH 8, 150 mM NaCl, 2 mM Na-EDTA) with 10 μg/ml pepstatin, 20 μg/ml aprotinin, 100 μg/ml leupeptin, 5 μg/ml E-64, and 0.25 mM phenylmethylsulfonyl fluoride (in early experiments leupeptin was 10 μg/ml and E-64 was not present). Large debris, nuclei, and mitochondria were removed by successive centrifugation at 600 and 10,000 × *g* for 10 min. The supernatant was mixed with one-fourth volume of TNE buffer with 5% Triton X-100, 2.5% deoxycholate, and 0.5% SDS (final concentrations: 1% Triton X-100, 0.5% deoxycholate, and 0.1% SDS). After 30 min on ice, the suspension was centrifuged at 160,000 × *g* for 15 min. The supernatant was transferred to a column containing 0.5 ml of immobilized preimmune IgG for pre-clearing, and the slurry was mixed by gentle rocking on ice for 30 min. The flow-through was applied to another column containing 0.5 ml of immobilized anti-NDP kinase antibodies, and incubated as above for 90 min. The effluent was drained and the immunoaffinity matrix was washed with 25 ml of RIPA buffer (TNE with 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS), 5 ml of TNE buffer, and 5 ml of 20 mM Tris·HCl, pH 6.8. Bound proteins were eluted with 2 ml of 0.1 M glycine, pH 3.0, precipitated with acid acetone, solubilized in 40 μl of urea sample buffer (4.6% SDS, 8 M urea, 150 mM Tris pH 8.0, 0.1% bromphenol blue, 50 mM DTT) by sonication, and alkylated with 100 mM iodoacetamide. Immunoaffinity columns were regenerated by extensive washing with 1 M NaCl, phosphate-buffered saline, and stored refrigerated in phosphate-buffered saline with 0.05% sodium azide. Prior to use, columns were washed with glycine buffer, 1 M NaCl, and RIPA buffer.

In control experiments, the post-mitochondrial fraction was isolated in 20 mM Tris, 2 mM EDTA buffer and brought to 5% SDS and 5 mM DTT (14). The mixture was boiled for 5 min, cooled, and equilibrated in RIPA buffer by gel filtration. The denatured lysate was centrifuged at 160,000 × *g* for 15 min and processed as above. In another set of control experiments, 20 μg each of CK, PK, GAPDH, and vimentin were dissolved in 6 ml of RIPA containing 2.4 mg/ml bovine serum albumin, applied to the antibody columns, and treated as described above.

Preparation of Crude Intermediate Filament Proteins from Frog Skeletal Muscle—Intermediate filaments (IF) were isolated by the procedure of Geisler and Weber (15) with the following modifications: 25 g of frozen leg muscle were homogenized in 300 ml of 50 mM MOPS, pH 7.0, with NaOH, 2 mM EDTA, 1 mM DTT, 0.6 M KCl, 1% Triton X-100, 1 μg/ml each leupeptin and E-64, 0.25 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine. The suspension was stirred on ice for 30 min and centrifuged for 10 min at 14,500 × *g*. Pellets were rehomogenized and extracted twice with this buffer. The pellets were then homogenized in 50 mM MOPS, pH 7.0, with NaOH, 1 mM EDTA, 1 mM DTT, 0.5% Triton X-100, 1 mM benzamidine, and extracted twice with this buffer, first for 2 and then for 6 h. The pellets were suspended in 50 mM Tris·HCl, pH 7.5, 6 M urea, 5 mM EDTA, and 5 mM DTT, stirred overnight, and centrifuged at 27,000 × *g* for 30 min. The pellets were re-extracted with urea buffer for 1 h and spun at 48,000 × *g* for 30 min. The combined supernatants were used as a crude IF fraction.

Microsequencing—After SDS-PAGE (4–15 or 4–20% gels), proteins were stained with Coomassie Brilliant Blue. Individual bands were excised and analyzed by mass spectrometry with a Finnigan-MAT TSQ7000 liquid chromatography-electrospray tandem quadrupole mass spectrometry system. Sample preparation, including in-gel tryptic digestion, was performed using a protocol developed by Kinter *et al.*[2] The tryptic digests were analyzed by capillary liquid chromatography (LC)-electrospray mass spectrometry (ESI-MS) to measure the molecular weights of the peptides present. Peptide sequences were determined by collisionally activated dissociation (CAD) using LC-electrospray tandem mass spectrometry (ESI-MS-MS). Peptide masses and partial sequences were utilized to search theoretical tryptic digest peptide mass and protein sequence data bases using the programs MSTag, BLAST, and ATLAS.

RESULTS

We utilized immunoaffinity methods to isolate and identify proteins associated with NDP kinase in cardiac muscle. Post-mitochondrial fractions from whole frog hearts were solubilized in RIPA buffer, precleared with immobilized preimmune IgG, and adsorbed to immobilized antibodies to frog NDP kinase. After stringent washes, bound proteins were eluted at low pH and resolved by SDS-PAGE in gradient gels. Analysis of stained gels revealed the presence of NDP kinase as a single band with an apparent mass of 18 kDa (Fig. 1*A*) and several other polypeptides. Of these, only 5 polypeptides with apparent masses of 28, 40, 45, 55, and 60 kDa were present in amounts appropriate for microsequencing. This pattern was seen with extracts from six hearts processed separately by the same protocol; columns with affinity purified IgG or the whole IgG fraction of immune serum yielded indistinguishable results. These proteins were not retained by immobilized preimmune IgG (Fig. 1*B*), and in immunoblots of the eluted fractions the antibody to NDP kinase did not recognize polypeptides with these molecular masses (see below). Furthermore, silver staining of gels shows no bands aside from NDP kinase in eluates from preparations that were heat-denatured in the presence of SDS prior to immunoaffinity purification (Fig. 1*B*). Gel staining and immunoblotting indicate that recovery of NDP kinase in denatured preparations was somewhat lower than in native samples (Figs. 1*B* and 3); this is expected, since the enzyme from frog tissues tends to aggregate when heated in SDS.[3] Nevertheless, the NDP kinase band was still clearly detectable in these eluates. This result demonstrates that the copurification of the other proteins with NDP kinase depends on native structures; furthermore, when denatured, these proteins do not share epitopes with NDP kinase and do not bind directly to the antibodies. The identity of the proteins that copurify with cardiac NDP kinase was investigated directly by microsequencing; the results obtained are summarized in Table I.

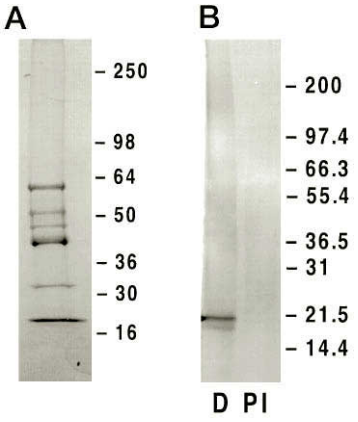

FIG. 1. **Immunoaffinity purification of NDP kinase from heart.** *A*, a heart muscle fraction eluted from columns of immobilized antibodies to NDP kinase was subjected to SDS-PAGE in a 4–15% gel and stained with Coomassie Brilliant Blue. *B*, eluates from a column of preimmune IgG (*PI*), or from a SDS-boiled lysate loaded on immobilized antibodies to NDP kinase (*D*) were analyzed by SDS-PAGE on a 4–20% gel and stained with silver. The positions of molecular mass standards are indicated at the sides.

Data base searches revealed that two of the partial peptide sequences obtained from the 28-kDa band show homology to members of a family of antioxidant proteins of molecular mass 20–29 kDa present in organisms ranging from bacteria to mammals (16). The highest sequence similarity was found with the product of the murine *MER5* gene, which is preferentially expressed in erythroleukemia cells (17). A third peptide obtained from the 28-kDa protein shows homology with a gag-like protein from *T. cruzi* (18). Since the MER5 antioxidant protein family is classified within the gag-akt polyprotein superfamily, the protein copurifying with NDP kinase may be a novel mem-

[2] M. Kinter, N. Sherman, and J. W. Fox, manuscript in preparation.

[3] A. S. Otero, unpublished results.

TABLE I
Microsequencing of proteins retained by immunoaffinity columns

Protein	Apparent M_r	Peptide data by CAD (measured M_r)[a,b]	Peptide data from data base (M_r)	Data base/accession[c]
NDP kinase	18,000	TFXAXKPDGVQR (1345.6) GDFC*XQVGR (1051.8) _ _NGNXXK (828)	TFIAIKPDGVQR (1344.8) GDFCIQVGR (1052.5) GLIGDIIK (828.5)	GenBank X97899 X97900
Unknown (MER5 homolog)[d]	28,000	PAVTQXA_ _ FK (1199.6) _ _ AVVN---K (1022.6) HFSGXXDhcR (1241.6)	PAVTQHAPYFK[1] (1239.6) GTAVVNGEFK[1] (1002.5) GFSGLLDHCR[2] (1161.6)	GenBank M28723[1] PIR 1078716[2]
GAPDH[d]	38,000	VVDXTVR (801.2) VXPSXNGK (828) XTGM$_{(o)}$AFR (811.4) VNXGXDGFGR (1025.8) VVDNXVYVASK (1027.6) VPTPNVSVVDX---R (1496.0)	VVDLTCR (864) VIPELNGK (870) ITGMAFR (812) VKVGINFGC (1032.5) VVDLCHMASK (1240.6) VPTPSVVDLTCR (1558.8)	GenBank U41753
Creatine kinase	45,000	XEEXFK (778.4) FEEXXTR (907.6) GGDDXDPNYVXSSR (1508.5) AXENXSXK (881.1)	IEEIFK (778.9) FEEILTR (908) GGDDLDPNYVLSSR (1508.6) AVEKLSIQ (868.5)	PIR 539482
Vimentin	53,000	FANFXDK (854.1) EYQDXXNVK (1123) M$_{(o)}$AXDXEXATYR (1311.8)	FANFIDK (854.4) EYQDLLNVK (1122.3) MALDIEIATYR (1311.7)	Swissprot P24789 P24790
Pyruvate kinase	60,000	VVPVP (510.6) APXXAVTR (839.6) gxFPVXYK (937.0) _ _YPXEAVR (1019.8) _ _XGXEXP_ _K (1142.2) XDXDSEPXTAR (1229.4) XYVDNGXXSXNVK (1448.6) Uninterpretable (1837.7)	VVPVP (510.7) APIISVTR (872) GIFPVLYR (965.2) GDYPLEAVR (1020.1) GDLGIEIPAEK (1142.2) LDIDSEPIVAR (1228.4) IYVDDGLISLLVK (1448.7) RFDEILEASDGIMVAR (1839.1)	GenBank U03878

[a] X designates I or L, which cannot be distinguished by low energy CAD; M$_{(o)}$ is oxidized M; lower-case letters indicate tentative assignments; _, designates a single unknown aminoacid; ---, designates an unknown number of unknown aminoacids; C*, designates a carbamidomethyl-modified cysteine residue.
[b] $M + H^+$.
[c] Data base information comes from sequences determined for *Xenopus laevi* proteins, with the exception of the 28-kDa protein, where sequences marked with superscript 1 are from murine MER5, and the sequence marked with superscript 2 is from the gag-related protein from *Trypanosoma cruzi*.
[d] Sequences from two independent experiments.

ber of this group of proteins. On the other hand, it is possible that the two groups of sequences obtained from this band reflect the presence of two different polypeptides that happen to comigrate in these gels.

Attempts to identify the 40-kDa protein using the molecular mass of peptide fragments were fruitless: mass mapping did not retrieve any proteins that could generate this series of peptides. This was also true when microsequencing was repeated using a second sample from another heart. However, searches of available data bases with the partial peptide sequences from this protein found high homology to GAPDH (40 kDa) from several species. While all of the peptides display high homology to all GAPDHs, there are some differences from the sequences of known forms of this enzyme, including the isoform cloned from *Xenopus* (GenBank, accession number U41753). In particular, some of the peptides identified through sequencing are not preceded by tryptic cleavage sites in any of the GAPDH sequences available in data bases, suggesting that this protein may be a novel isoform of GAPDH. This finding accounts for the lack of similarity in the mass of some tryptic peptides obtained here to those predicted from the primary structure of known GAPDHs.

The 45-kDa band was readily identified as CK, based on the size and sequence of tryptic peptides. Two of the peptides obtained, IEEIFK and FEEILTR, are specific for the muscle (M) isoform of CK, and come from highly conserved regions of this protein. The third sequence, GGDDLDPNYVLSSR, is found in both muscle and brain CK. These peptides show 100% identity to the *Xenopus* type III enzyme expressed in muscle (19). The fourth peptide differs somewhat from the analogous sequence in *Xenopus* type III CK, but still displays 62.5% homology. These differences are not unexpected, since the CK isozyme repertoire of *Xenopus* differs markedly from other amphibians such as *Rana* (19).

The information obtained from the polypeptide with molecular mass of 55 kDa allowed its unambiguous identification as vimentin (20). These sequences come from highly conserved regions found at the amino and carboxyl terminus of the central rod domain shared by all IF proteins. The first one, FANFIDK, comes from the beginning of helix 1A, while the second and third peptides are adjacent and located near the end of helix 2B of the rod domain (21).

Mass matching and partial peptide sequences from the 60-kDa band predicted its identity as an M2 isoform of PK, with high homology to the *Xenopus* PK (22). Since PK is conserved throughout evolution, this high degree of homology extends to the mammalian enzymes as well. Note that even a peptide with an uninterpretable sequence could be assigned to PK by mass matching (Table I).

The identity of proteins associated with NDP kinase, with the exception of the 28-kDa polypeptide (for which there are no commercially available antibodies), was confirmed by immunoblotting of eluates obtained under identical conditions. To reduce degradation of vimentin, homogenization and wash buffers were supplemented with the protease inhibitor E-64 and the concentration of leupeptin was increased by 10-fold. Fig. 2*A*

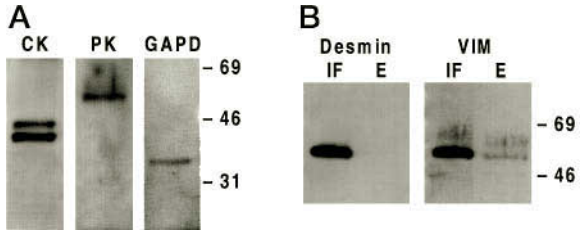

FIG. 2. **Immunoblot analysis of proteins that copurify with NDP kinase.** *A*, an eluate obtained as in Fig. 1*A*, except for increased amounts of leupeptin and the presence of E-64 (see "Methods") was resolved in 4–15% gels and transferred to nitrocellulose. Membranes were probed with antibodies to CK, PK, and GAPDH. *B,* reactivity of a crude intermediate filament fraction from skeletal muscle (*IF*) and proteins eluted from immunoaffinity columns to NDP kinase (*E*) with an antibody to desmin (*left panel*). The same membrane was reprobed without stripping with an antibody to vimentin (*VIM; right panel*). The positions of molecular mass standards are indicated on the *right side*.

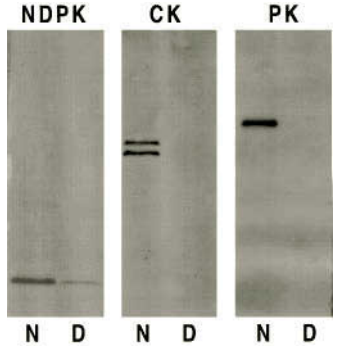

FIG. 3. **Comparison of eluates obtained from native and denatured cardiac muscle extracts.** RIPA extracts (*N*) and SDS-boiled lysates (*D*) from cardiac muscle were subjected to immunoaffinity purification as described under "Methods," resolved in 4–15% SDS gels, and transferred to nitrocellulose. Strips of the membrane were probed with antibodies to NDP kinase (*NDPK*), CK, and PK.

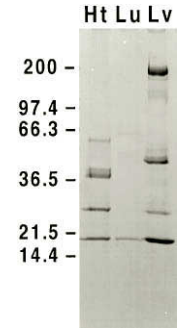

FIG. 4. **Proteins associated with NDP kinase in different tissues.** RIPA extracts from heart (*Ht*), lung (*Lu*), and liver (*Lv*) were subjected to immunoaffinity purification under identical conditions. Eluates from immunoaffinity columns were resolved in 4–20% gels which were stained with Coomassie Brilliant Blue. The positions of molecular mass standards are indicated on the *left*.

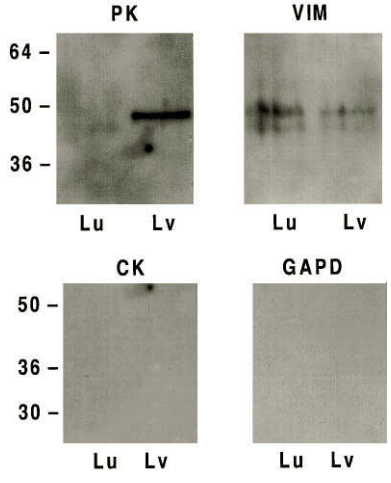

FIG. 5. **Immunoblot analysis of eluates from lung and liver.** Samples from lung (*Lu*) and liver (*Lv*) were obtained as described in the legend to Fig. 4, resolved in 4–15% gels, and transferred to nitrocellulose. Membrane strips were immunoblotted with antibodies to PK, vimentin (*VIM*), CK, and GAPDH.

shows that eluates do contain PK, GAPDH, and CK, which migrate with the apparent masses expected from the stained gel pattern. In immunoblots CK ran as a doublet, due to the presence of the muscle and brain isoforms in heart muscle (19). Since the major IF protein present in adult cardiac muscle is desmin and not vimentin (21, 23), blots were initially probed with a monoclonal antibody to this protein. Fig. 2*B* shows that this antibody cross-reacts with desmin in crude IFs isolated from frog skeletal muscle, but not with eluates. In contrast, when the immunoblots were re-probed with antibodies to vimentin, a broad band was detected in eluates and in the IF fraction as well (Fig. 2*B*). Vimentin was detected as a broad band at 58 kDa, with variable amounts of lower mass (55 and 52 kDa) polypeptides. This heterogeneity results from the activity of an ubiquitous calcium-activated thiol protease that targets IF proteins (24). Immunoblotting of the eluate shown in Fig. 1*A* with the antibody to vimentin revealed that the upper band was much less prominent in that sample (not shown), while the 55 kDa gave a strong signal. Presumably, incomplete inhibition of this protease in earlier experiments led to the degradation of vimentin.

When the same set of antibodies was utilized to probe immunoblots of SDS-boiled eluates no bands were detected. While NDP kinase was evident in both native (N) and denatured (D) extracts, Fig. 3 shows that CK and PK were absent from denatured preparations. The same pattern was observed with GAPDH and vimentin (not shown). Purified vimentin, CK, PK, and GAPDH were not retained by the immunoaffinity matrix (not shown). Thus, these proteins bind to anti-NDP kinase affinity columns indirectly, through specific, denaturation-sensitive interactions with cellular NDP kinase.

To determine whether there is any tissue specificity in the formation of complexes between NDP kinase and other proteins, the protocol utilized for affinity chromatography of heart muscle extracts was applied to frog lung and liver. Comparison of the composition of eluates obtained from heart, lung, and liver demonstrates significant differences on polypeptide patterns (Fig. 4). The differences between tissues were confirmed by immunoblotting (Fig. 5). Eluates from liver contain vimentin and PK, but not CK or GAPDH. Eluates from lung contain only vimentin, but not PK, GAPDH, or CK. The absence of CK in liver is expected, since this tissue does not normally express CK (25, 26); however, lung expresses CK, and both tissues express the muscle isoform of PK (27). As to GAPDH, this housekeeping enzyme is expressed constitutively in all tissues (28), being routinely used as a control for RNA loading in Northern blots. Therefore, energy metabolism enzymes associate with NDP kinase in what is truly a tissue-specific manner, that is, not dictated by their expression patterns.

The 28-kDa protein was present in lung and liver eluates, as identified by its comigration with the polypeptide found in heart (Fig. 4). As to vimentin, the amounts detected by immunoblotting are in agreement with the expression levels in lung and liver. In adult liver vimentin expression is limited to a few cell types (29). In contrast, lung expresses high levels of vimentin (30). Indeed, immunoblotting with antibodies to vimentin shows a stronger signal in eluates from lung than in those

obtained from liver (Fig. 5), despite the lower amounts of total protein in lung samples (Fig. 4).

DISCUSSION

The present work reports the identification of 5 polypeptides that copurify with cardiac NDP kinase. Association between these proteins and NDP kinase is presumably the result of relatively strong protein-protein interactions and requires native structures, since these complexes survive stringent washes with detergent-containing or low osmolarity buffers, but are disrupted by denaturation.

The finding that vimentin co-purifies with NDP kinase from cardiac tissue was totally unanticipated, since under our isolation conditions IFs are mostly insoluble. Nevertheless, the source of the vimentin associated with NDPK might be a small pool of soluble oligomers found in other cells (reviewed in Ref. 21). Moreover, the primary IF protein of mature myocytes is desmin, which is present in large excess over vimentin in muscle cells (21, 30). Cardiac myocytes probed with anti-vimentin antibodies show a small amount of staining,[3] which contrasts with a strong reactivity for desmin (23). Thus, association of vimentin with heart NDP kinase with the exclusion of the prevalent desmin suggests that this interaction is highly specific.

Vimentin and the 28-kDa protein were found in association with NDP kinase in lung, liver, and heart (Figs. 2*B*, 4 and 5); thus, this interaction may be essential for the function of all three proteins. Extension of these observations to other tissues and investigation of the functional outcome of complex formation between these proteins are in progress, to verify this hypothesis. The connection between NDP kinase and vimentin is of special interest, in view of the reported links of both proteins to the progression and invasiveness of certain types of cancer (2, 31–34). For instance, this interaction could account for the link between NDP kinase expression and alterations in tumor cell motility (33). Likewise, the MER5 homolog protein described here may be identical to a 28-kDa protein that coprecipitates preferentially with the $Ser^{120} \rightarrow Gly$ mutant of NDP kinase A found in neuroblastoma (34); if so, this association could also be relevant to tumor progression.

In contrast to vimentin and the 28-kDa protein, the association of PK, CK, and GAPDH with NDP kinase was found to be tissue-specific (Figs. 4 and 5). This specificity may be caused by changes in the compartmentalization of these enzymes, variations in their primary structure, or by different patterns of expression of NDP kinase isoforms (11). Nevertheless, it suggests that NDP kinase plays distinct roles on the energy metabolism of each of these organs. Since PK, CK, and GAPDH reportedly associate with vimentin IFs (35, 36), it is possible that the interaction between NDP kinase and the other three enzymes is indirect and depends on the formation of individual complexes of each of these proteins with vimentin, with the latter process being endowed with tissue specificity. This hypothesis is attractive, since it casts vimentin-containing IFs in the role of the structural element involved in the compartmentalization of enzymes involved in ATP regeneration, such as NDP kinase and CK, or in ATP formation, such as PK and GAPDH. Additionally, it is tempting to speculate that the role of the 28-kDa homolog of antioxidant proteins in these complexes would be to preserve the integrity of vimentin filaments, which are sensitive to oxidative damage (37). Nevertheless, PK was previously found to associate directly with NDP kinase (38), so it is equally possible that NDP kinase interacts with each one of these proteins independently and directly. Answers to this question will have to await the reconstitution of complexes of NDP kinase with highly purified proteins, and may require the identification of unknown components that might be essential for the formation of these putative multiprotein complexes.

In conclusion, the combination of immunoaffinity chromatography and microsequencing by mass spectrometric methods is clearly a powerful strategy that allows unambiguous identification of a number of proteins associated with NDP kinase. The major drawback of this approach is that proteins must be present in substantial amounts in eluates to be sequenced, so that polypeptides present at low abundance cannot be characterized. Therefore, it provides a far from complete portrait of the protein-protein interactions that underlie the multiple functions of NDP kinase in different cells and tissues. Nevertheless, this work is a necessary step toward the understanding of the physiological role of NDP kinase.

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