RESEARCH ARTICLE

Landscape of the hnRNP K protein–protein interactome

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The heterogeneous nuclear ribonucleoprotein K is an ancient RNA/DNA-binding protein that is involved in multiple processes that compose gene expression. The pleiotropic action of K protein reflects its ability to interact with different classes of factors, interactions that are regulated by extracellular signals. We used affinity purification and MS to better define the repertoire of K protein partners. We identified a large number of new K protein partners, some typically found in subcellular compartments, such as plasma membrane, where K protein has not previously been seen. Electron microscopy showed K protein in the nucleus, cytoplasm, mitochondria, and in vicinity of plasma membrane. These observations greatly expanded the view of the landscape of K protein–protein interaction and provide new opportunities to explore signal transduction and gene expression in several subcellular compartments.

Received: August 30, 2005
Revised: November 2, 2005
Accepted: November 2, 2005

Keywords:
Affinity chromatography / hnRNP K protein / Liquid chromatography-tandem mass spectrometry / Protein–protein interactions

1 Introduction

Heterogeneous nuclear ribonucleoprotein K (hnRNP K) is a conserved RNA/DNA-binding protein that is involved in multiple steps that compose gene expression and signal transduction [1–3]. K protein involvement in so many processes reflects its ability to directly interact with not only nucleic acids but with kinases and chromatin, transcription, splicing, and translation factors. Most of these interactions were previously identified in yeast two hybrid screens [4, 5], pull-down assays [6, 7], and immunoblotting [8–10].

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Abbreviations: IP, immunoprecipitation; **KI**, K protein interactive; **miRNA**, micro-RNAs; **SIC**, spreading initiation center

MS is a powerful proteomic tool to identify protein–protein interaction on a genome-wide scale [11–13]. We used affinity purification and MS analysis to gain a broader view of the repertoire of hnRNP K protein partners. In addition to several of the known partners we identified a larger number of new proteins that exist in dynamic complexes with K protein.

2 Materials and methods

2.1 Cells

Rat hepatoma cells expressing HTC-IR were grown in plastic cell culture flasks in DME media supplemented with 10– FBS, 2 mM glutamine, penicillin (100 U/mL), streptomycin (0.01%), and humidified with 7/93% CO_2/air gas mixture [14].

2.2 Purification of K protein

Two methods, immunoprecipitation and complexing to bacterially expressed K protein, were used for purification of K protein and its molecular protein partners.

HTC-IR cells were sonicated three times for 3 s on ice (model 130-W Cole-Parmer Ultrasonic Processor, set at amplitude 80) with 1.0 mL of immunoprecipitation (IP) buffer (150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% NP 40, 50 mM Tris-HCl, pH = 7.5) containing the following inhibitors: 10 μg/mL leupeptin, 0.5 mM PMSF, 0.5 mM DTT, 30 mM *p*-nitrophenyl phosphate, 10 mM NaF, 0.1 mM Na_3VO_4, 0.1 mM Na_2MoO_4, and 10 mM β-glycerophosphate and kept on ice for 15 min. Cell lysates were centifuged (15 000*g*, 4°C, 20 min); supernatants were diluted in 10 mL of IP buffer and run by gravity through four columns: three containing 1 mL of resin made up of sepharose conjugated with rabbit IgG and one column containing 1 mL sepharose conjugated with #54 antibody. Anti-K protein antibody #54 directed against the C-terminus or rabbit IgG were immobilized to the Protein A Sepharose using disuccinimidyl suberate (DDS) and Seize X Protein A immunoprecipitation Kit (Pierce). After running the load, columns were washed separately with IP buffer containing 200 mM NaCl. Then, proteins were eluted from resin firstly with IP buffer containing an appropriate salt concentration (0.6 and 1.0 M NaCl), followed with 0.1 M glycine, pH = 2.6.

To purify K protein by complexing to recombinant His-K, cDNA of K protein were subcloned into pET-28(+) expression vector (Novagen), and the plasmids were transformed into *Escherichia coli* BL21 DE3 pLysS cells (Novagen). Bacterial cells were harvested by centrifugation, and following freezing and thawing, the bacterial pellet was suspended in PBS containing 5 mM DTT, 0.1 mM leupeptin, 0.5 mM PMSF, 0.1 mM lysozyme, and sonicated on ice. After centrifugation, recombinant His-K protein was attached to His-binding resin (Novagen) in binding buffer containing 50 mM NaH_2PO_4, pH = 8.0, 300 mM NaCl, 10 mM imidazole, 10% glycerol, 20 mM β-mercaptoethanol, 2 mM ATP, and 10 mM $MgCl_2$. Then, the resin was washed extensively with binding buffer containing 2 M NaCl.

Cell lysates diluted in 10 mL of IP buffer were applied onto the four columns connected in series: three upper columns contained 1 mL of His-binding resin and one lower column contained 1 mL of His-K protein resin. After running the load, columns were washed separately with binding buffer containing 25 mM NaCl, and proteins were eluted from resin with binding buffer containing 0.2, 0.4, and 1.0 M NaCl.

Proteins eluted from the columns were concentrated by ultrafiltering the sample solution through an anisotropic membrane (regenerated cellulose, 3 000 MWCO) (Centricon Centrifugal Filter Devices, Millipore). To exchange buffer of protein samples, Protein Desalting Spin Columns (Pierce) were used.

2.3 MS

Protein samples were analyzed by LC-ESI-MS-MS/MS with collisional fragmentation [15]. Prior to analysis the proteins were reduced, alkylated (when necessary), and digested with trypsin (sequencing grade; Promega) or V8 protease or cyanogen bromide following a standard protocol. Eluted peptide mixture was applied to RP-18 precolumn (LC Packings) using water containing 0.1% TFA as mobile phase and than transferred to nano-HPLC RP-18 column (LC Packings, 75 μM id) using an ACN gradient (0—50% AcN in 30 min) in the presence of 0.05% formic acid with the flow rate of 200 nL/min. Column outlet was directly coupled to nano-Z-spray ion source of Q-Tof electrospray mass spectrometer (Waters) working in the regime of data-dependent MS to MS/MS switch, allowing for 3 s sequencing scan for each detected peptide. A blank run ensuring lack of crosscontamination from previous samples preceded each analysis. The output list of parent and daughter ions was compared with reference database of ions using MASCOT (MatrixScience) tool [16]. Peptide identification and the presence of their covalent modifications were verified by inspection of parent mass fragmentation patterns using programs MassLynx (Waters) and ProteinProspector.

2.4 Quantitation of protein levels

Relative levels of protein K binding proteins in different samples were compared by measuring their peptide peak areas in a survey scan normalized to the summed peak areas of endogenous protein K peptides. Peptides identified in a LC-MS-MS/MS run by MASCOT [16] program were localized by their m/z on a survey scan (no fragmentation) 2-D (retention time *vs.* m/z) maps [16]. In-house software was used to pick peaks corresponding to a given m/z in survey scan 2-D maps and for visual inspection of the data and verification of the results. Peak volumes were calculated by fitting the isotopic envelope expected for a given peptide to the raw data isotopic envelope, after background subtraction and low-pass filtering. In case of the presence of isobaric peptides in a single run, only one peptide with the best fit (minimum mean square error) to the expected isotopic envelope was selected. Peptides present in less than 50% of samples were not taken into account.

The results of the automatic search were viewed and evaluated, and if needed, corrected using the graphic interface of application. The manual corrections were done when low-abundance peptides were affected by high noise level or the spectrums of two peptides are superimposed. In order to obtain relative amounts of a single protein in all samples, the absolute areas of its peptides were divided by the mean area of the peptides of the K protein and then averaged. Before averaging, the relative areas were normalized over the samples to obtain equal contribution of each analyzed peptide. Then, the mean values and SDs of the relative protein binding levels in groups of samples were computed, and the

analysis of variance (ANOVA) was used to find if statistically significant differences between the groups existed. For each protein two tests of mean values equality were performed: between the starved cells group and all the remaining samples, and in all groups simultaneously (in this case tests for pairs of groups were also performed to determine which groups were different).

2.5 Immunogold electronmicroscopy

Immunogold labeling was done as previously described [17] (EMBS, Frederick, MD). Briefly, HTC-IR cell pellets were fixed in 4.0% paraformaldehyde in PBS. After mincing the cell pellet was washed with Millonig's PBS buffer. The pellets embedded in LR White plastic resin were ultrathin sectioned into 60–80 nm samples and were mounted onto 200 mesh nickel grids.

The sample grids were either incubated with anti-K antibody (#54) or preimmune serum (1:80 dilution) in PBS for 3 h at RT. After washes all sample grids were incubated with goat antirabbit colloidal gold (10 nm particles) (1:20 dilution) in PBS. After 2 h of incubation, grids were washed with PBS and water. The grids were poststained with 2% uranyl acetate and Reynold's lead citrate. The grids were examined using JEOL 1200 EX Transmission Electron Microscope.

2.6 Pull-down reaction

The PCR-generated full-length cDNA of human Grb2 [18] and its fragment (between 163 and 396 nucleotide) were subcloned into pGEX 4T-1 (Pharmacia Biotech), and the plasmids were transformed into *E. coli* BL21 DE3 pLysS cells (Novagen). Bacterially expressed recombinant proteins were purified by affinity chromatography using gluthatione agarose beads. The labeled hnRNP K protein was prepared by the translation of the *in vitro* transcribed K protein mRNA in the reticulocyte lysate translation system in the presence of [^{35}S]methionine using the TNT T7 Quick Coupled Transcription/Translation System as per the manufacturer (Promega). The pull-down reactions were performed exactly as described [19].

3 Results

3.1 MS-based identification of K protein's complexes components

Identification of molecular partners represents a powerful tool to explore proteins function. K protein is thought to participate in a host of cellular processes [1–3]. Yet many details remain to be defined with respect to not only its function in specific intracellular processes but also K protein's potential contribution to shaping protein–protein network topology.

To gain broader insight into K protein's function we used affinity purification [20] and MS (Fig. 1). In the preliminary experiments, protein complexes eluted from the affinity columns were separated by 2-DE; silver-stained protein spots were excised and subjected to in-gel reduction, alkylation, and tryptic digestion followed by MS identification. MS analysis of the proteins specifically immunoprecipitated by anti-K proteins antibody that were separated by 2-DE unexpectedly revealed that majority of protein spots represented different fragments of K protein or proteins nonspecifically bound to preimmune column (not shown), and only three spots were those potentially interacting with K protein. Next we used MS to analyze 2DE spots representing proteins

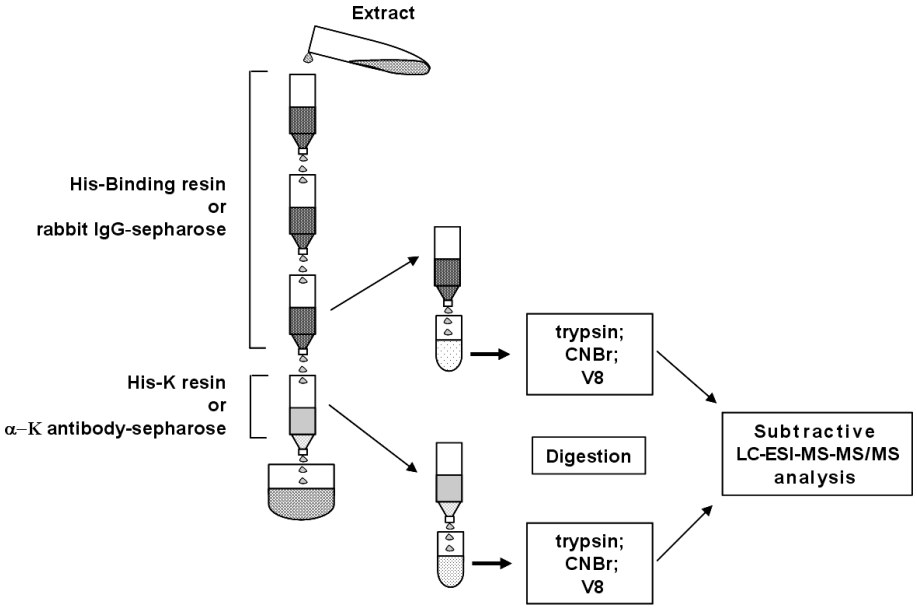

Figure 1. Affinity purification and MS large-scale strategy to identify hnRNP K interacting proteins. Extracts from HTC-IR cells were applied onto the four of tandem columns connected in series: three upper columns contained His-binding resin (or rabbit IgG sepharose) and the bottom column contained His-K protein resin (or anti-K protein antibody-sepharose). After loading extracts, the columns were washed separately. Proteins eluted individually from the third and the bottom columns were digested and then analyzed separately by LC-ESI-MS-MS/MS.

purified by His-K affinity column. This allowed us to identify nine other proteins that interact specifically with K protein, but again, the majority of the spots found on gels represented proteins that nonspecifically attached to the column resin support (not shown). Thus, protein spots on gels that were bound nonspecifically to preimmune agarose and His-binding resin represented fragments of K protein and made identification of protein spots of interest difficult.

To improve identification of K protein partners we chose to use affinity purification [20] and MS analysis without prior 2-DE separation (Fig. 1). Subsequently, we performed several independent experiments for each of two isolating procedures using module columns with either His-K bait or antibody directed against K protein. Protein complexes eluted from the affinity columns were subjected to reduction, alkylation, and tryptic digestion followed by MS identification. Proteins were considered to be specifically bound by K protein's complexes if they were eluted from the bottom of either the His-K or anti-K modules but not from any of the corresponding top precolumns. Each one of the proteins was identified by at least two unique peptides that were manually verified to minimize false-positive identification. The list of proteins presented in K protein's complexes, and their Gene Ontology [21] descriptions are included in Suppl. Tables 1–3.

Expectedly, the proteins bound by K protein were found in different amounts (not shown). Some of them were represented in MS assay by multiple peptides in most of analytical runs, whereas others in individual runs by singular peptides. Unexpectedly, lists of proteins that interacted with K protein differed depending on the method used for isolation of the K protein's complexes. From 72 proteins isolated by His-K columns and 42 proteins isolated by immunoaffinity columns, only 13 proteins (Suppl. Tables 1–3) were common for both protein lists. Possible reasons for different identities and quantities of proteins found by the two methods may reflect PTM of the endogenous hnRNP K purified with its partners by the antibody columns.

When the modules containing K protein's complexes were treated with RNase (50 μg RNase A/mL, 25°C for 30 min), six proteins, three acidic ribosomal proteins (Arbp, Rplp2, Rplp1), CacyBP, Cct5, and Hnrph protein were removed from the complexes. Elution of these proteins with RNase suggests that these factors were associated with K protein *via* RNA.

Of 114 proteins isolated from hnRNP K protein complexes in our study, 22 proteins were previously identified as components of Grb2-SH2 complex [13] and 29 proteins were found in spreading initiation center (SIC) [22] or as interacting with talin, vanculin, or paxillin, proteins that compose SIC structure (Suppl. Tables 1–3). Some of them, including hnRNP K protein, were common for both Grb2-SH2 and SIC complexes.

Factors forming multiprotein complexes can interact directly or indirectly with scaffold proteins such as hnRNP K. As reported recently [23], the PPNP amino acid sequence of p21-activated kinase 1 (Pak1) binds directly the SH3 domain of the Grb2 adapter protein. The PPNP sequence is also found within hnRNP K (ScanSite Program) [24] (Fig. 2A). To confirm the interaction between hnRNP K and Grb2, we used ^{35}S-hnRNP K protein binding in GST pull-downs. As shown in Fig. 2B, ^{35}S-hnRNP K protein binds to full-length Grb2 protein and, to lesser extent, to its SH2 fragment (55–132 aa). There was no binding to GST alone. This experiment provides evidence for direct interaction of K protein and Grb2.

3.2 Dynamics of complexes containing K protein

The mitogen-induced transition from quiescent to proliferating state offers a rich environment to explore pathways and factors that mediate gene expression. Furthermore, because there is a nuclear shift of K protein in states of enhanced proliferation [25] understanding the role of K protein in mitogenic responses is relevant.

Exponentially growing HTC-IR cells were made quiescent by 48 h serum deprivation and then were treated with 15– FBS for 0, 1, 3, 6, and 24 h. Cellular extracts in IP buffer were purified using His-K protein tandem affinity column. Columns were extensively washed with binding buffer containing 25 mM NaCl to remove proteins that interacted nonspecifically with column's support, and the remaining proteins were eluted from the beads with binding buffer containing 0.2, 0.4, and 1.0 M NaCl. Proteins eluted from the control (His-resin only) and affinity columns (His-K) were used for analysis by MS without 2-DE separation.

Computing of the peptide peak areas of proteins bound by K protein showed nine proteins with relatively stable levels within the complex, six proteins with decreased levels, and three other proteins with significantly increased association within hnRNP K complex (Table 1). Although the biological significance and the molecular mechanisms of these findings remain to be defined, changes in composition of K protein's complexes in response to serum induction may reflect not only changes in the affinity of these interactions but also different expression and cellular localization of endogenous hnRNP K or/and its partners.

We also found 11 proteins (hnRNP L protein, DDX5, DDX17, ELAVl1-Hur, RPL13, IPLA2γ, Slingshot homolog 1, ACTN4, LMNA, ACTG1, capping protein) associated with K protein only when the complexes were isolated from proliferating cells. This could indicate that either there is increased interaction with hnRNP K or expression of these proteins in proliferating cells is increased, or both. To differentiate between these possibilities equal amounts of proteins from lysates of quiescent and proliferating cells were subjected to reduction, alkylation, and tryptic digestion followed by gel-free shotgun LC-MS-MS analysis. This analysis revealed equal levels of peptides representing these proteins in lysate from both resting and proliferating cells (not shown), indicating equal levels of proteins' expression. Taken

Table 1. List of K protein partners identified in resting and proliferating cells. Exponentially growing HTC-IR cells were serum starved for 48 h and then the cells were treated with 15% FBS. Then, the cells were lysed in IP buffer, lysates were loaded onto K-His columns, and eluted proteins were analyzed using MS. Relative levels of protein K binding proteins in different samples were compared by measuring their peptide peak areas in a survey scan normalized to the summed peak areas of protein K peptides. Results are expressed as the fold change of control cells (mean) yield of three independent assays for each time point measured (SD for each time point in brackets)

Protein	Serum, h					
	0	1	3	6	24	Comparison[a)]
C1qbp	1.0000 (22.42%)	1.9529 (16.05%)	2.5767 (25.66%)	2.3744 (8.80%)	N.D.	↑[b), c)]
Rps19	1.0000 (15.21%)	0.5801 (6.17%)	0.579 (8.71%)	0.4563 (19.47%)	N.D.	↓[b), c)]
Rpl23a	1.0000 (17.99%)	0.7724 (3.56%)	0.7119 (14.25%)	0.6232 (17.78%)	N.D.	↓[b), c)]
H2b	1.0000 (4.39%)	0.9135 (7.23%)	0.7993 (11.93%)	0.8071 (3.89%)	N.D.	↓[b), c)]
H2a	1.0000 (14.38%)	1.1189 (9.72%)	0.7317 (40.00%)	0.4944 (15.63%)	N.D.	↓[c)]
Rpl9	1.0000 (12.19%)	1.3036 (26.28%)	1.2707 (26.33%)	1.096 (9.48%)	N.D.	↔
Rps5	1.0000 (28.35%)	0.7951 (13.51%)	1.0089 (37.59%)	0.995 (36.85%)	N.D.	↔
Sfpg	1.0000 (15.28%)	0.9719 (22.31%)	0.9084 (35.59%)	0.6907 (1.31%)	N.D.	↔
Rps25	1.0000 (14.68%)	0.635 (13.08%)	0.6468 (15.86%)	0.5529 (14.75%)	N.D.	↓[b), c)]
Rps8	1.0000 (13.38%)	0.7773 (18.88%)	0.6151 (5.41%)	0.5455 (28.58%)	N.D.	↓[b), c)]
Rps3	1.0000 (8.89%)	0.8862 (8.06%)	N.D.	N.D.	0.8615 (22.45%)	↔
Pdip46	1.0000 (15.17%)	1.2935 (11.19%)	N.D.	N.D.	1.7832 (12.44%)	↑[b), c)]
Hnrpa2b1	1.0000 (19.32%)	1.0682 (26.53%)	N.D.	N.D.	2.1478 (11.37%)	↑[c)]
Rps4	1.0000 (18.81%)	0.8253 (27.86%)	N.D.	N.D.	1.0458 (34.40%)	↔
Ddx1	1.0000 (28.62%)	1.0573 (25.35%)	N.D.	N.D.	1.1348 (11.86%)	↔
hnrpg	1.0000 (14.86%)	0.9108 (7.43%)	N.D.	N.D.	1.0697 (32.39%)	↔
Xrn2	1.0000 (9.29%)	1.2 (28.48%)	N.D.	N.D.	1.537 (47.46%)	↔
CGI-99	1.0000 (28.89%)	0.7197 (21.85%)	N.D.	N.D.	1.537 (47.46%)	↔

a) ↔: No significant changes in the peptide peak areas compared to starved (control) cells; ↓/↑: Statistically significant decrease/increase in the peptide peak areas compared to starved (control) cells.
b) Statistically significant differences ($p < 0.05$) between the starved cell group and all the remaining samples.
c) Statistically significant differences ($p < 0.05$) in all groups simultaneously.

A

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                          20        30        40        50        60
                                                ****  Grb2 binding/interacting site
p21-activated kinase 1  PPAPPMRNTSTMIGAGSKDAGTLNHGSKPLPPNPEEKKKKDRFY-RSILPGDK
                        :: :: :.     : :.. : .:     :::: :  .      : :   :::.
        hnRNPK protein  PPPPPGRG-----GRGGSRARNL-----PLPPPPPRGGDLMAYDRRGRPGDR

                    290           300           310       320       330
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B

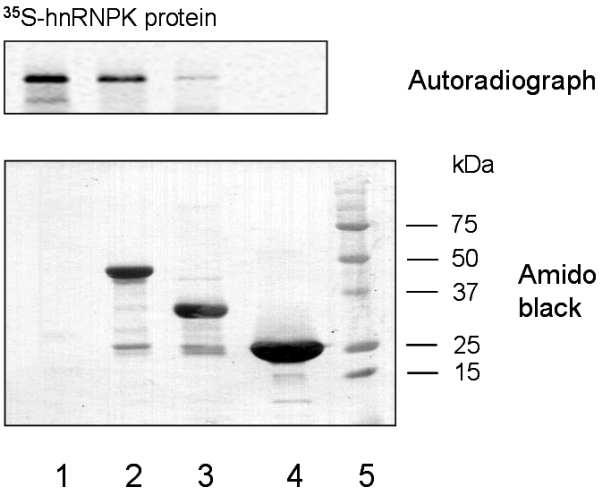

Figure 2. Direct interaction of hnRNP K with Grb2 adapter protein *in vitro*. (A) Comparison of hnRNP K protein and p21-activated kinase (PAK1) [23] identified a common PPNP amino acid sequence (ScanSite program) [24]. (B) Purified GST, GST-SH2-Grb2, and GST-Grb2 proteins bound to gluthatione beads were incubated with *in vitro* translated ^{35}S-hnRNP K protein at 30°C for 30 min, and the pull-down assays were performed as described previously [19]. Proteins eluted from the beads were analyzed by SDS-PAGE, transferred to PVDF membrane, and visualized by autoradiography (*Autoradiograph*) using Molecular Imager FX Pro Plus (BioRad) and protein staining (*Amido black*). 1 – ^{35}S-hnRNP K protein input material (1/20 volume used for pull-down reaction); 2 – GST-Grb2; 3 – GST-SH2-Grb2; 4 – GST; 5 – protein standard.

together, these studies show that K protein is a component of dynamic complexes whose composition is changing in response to mitogenic signaling.

3.3 Electron microscopy

K protein is known to interact with a diversity of cytoplasmic and nuclear factors, observations that are consistent with its well-described localization in these two major subcellular compartments [9, 25, 26]. Our MS analysis for the first time uncovered K protein partners that are typically found associated with plasma membranes and mitochondria (Fig. 3, Venn diagram), intracellular regions where K protein has not previously been shown to exit. We used immunogold labeling and EM [17] to better define subcellular location of K protein. The EM of grids incubated with anti-K protein antibody revealed gold particles not only in the nucleus and cytoplasm, but also within mitochondria and in the vicinity of plasma membrane (Fig. 4A–C). No gold particles were seen in the grids incubated with preimmune serum (Fig. 4D). The EM studies revealed that K protein is found in multiple subcellular compartments including the vicinity of plasma membrane.

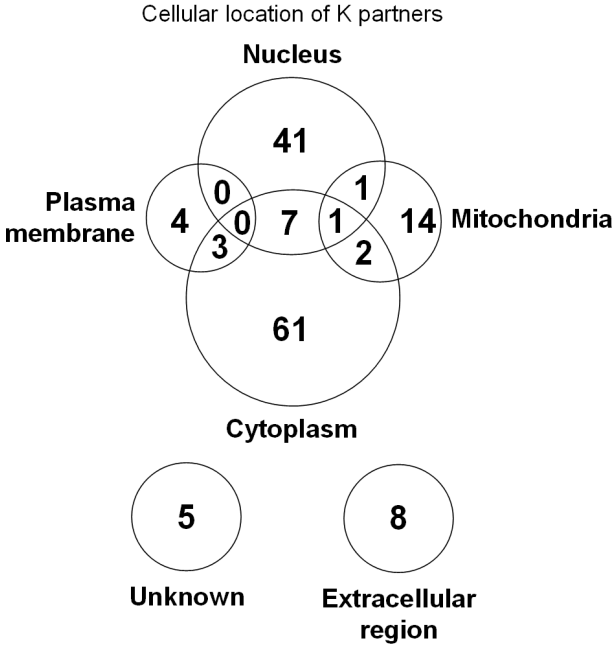

Figure 3. Venn diagram shows the intracellular distribution of hnRNP K protein-associated factors.

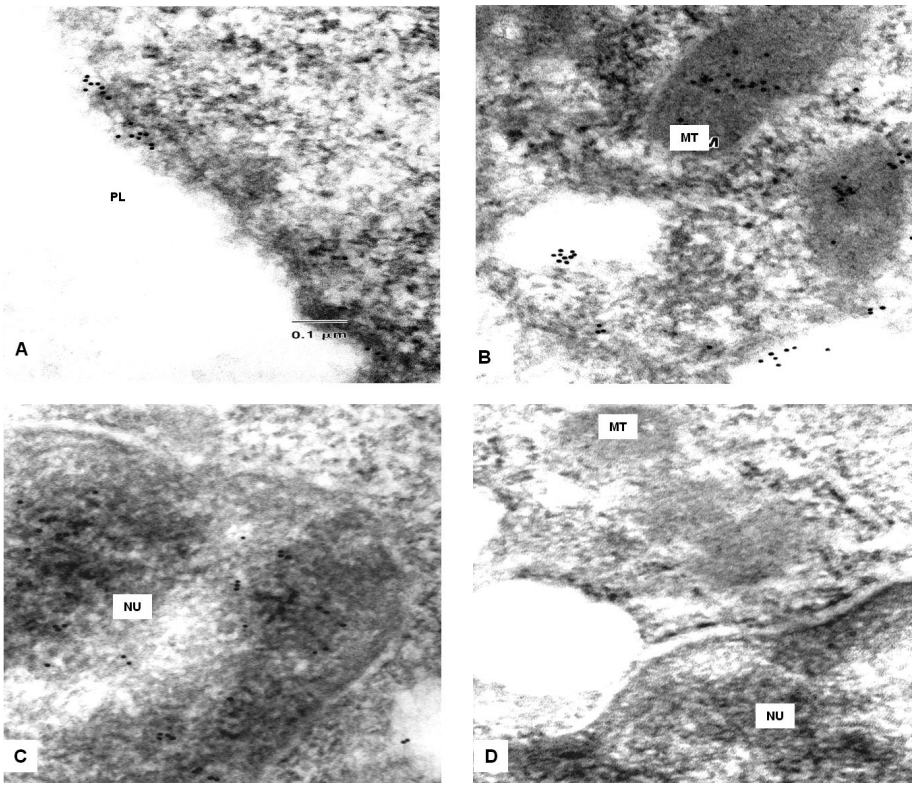

Figure 4. Intracellular localization of hnRNP K protein using immunogold labeling and electron microscopy. Proliferating HTC-IR cells were fixed in 4.0% paraformaldehyde in PBS. Cell pellets embedded in plastic resin were ultrathin sectioned and were mounted onto nickel grids. The sample grids were incubated with either anti-K antibody (#54) (A–C) or preimmune serum (D) and with goat antirabbit colloidal gold. After poststaining grids were examined using JEOL 1200 EX transmission electron microscope. PL, plasma membrane; MT, mitochondria; NU, nucleus.

4 Discussion

We used affinity purification and MS (Fig. 1) to gain a broad view of the repertoire of hnRNP K protein partners. Consistent with its role in multiple processes [1–3] we identified known and new K protein-associated factors (Suppl. Tables 1–3) that participate in many steps that compose signaling cascades and gene expression in several subcellular compartments (Fig. 5).

4.1 Nucleus

K protein is most abundant in the nucleus where it colocalizes with chromatin [27–29]. K protein interacts with the chromatin remodeling factor Polycomb Group (PcG) factor Eed [30]. Eed exists in a complex with Ezh2, a histone methyltransferase (HMT), methylating H3 at K9 and K27 [31]. In yeast two-hybrid system K protein also interacts with DNA-methyltransferases and scaffold attachment factor–B (SAF-B) [32], another component of the nuclear matrix. Consistent with these observations we found that K protein is a component of complexes that included histones, interactions that respond to extracellular signals (Table 2). The interaction of K protein with histones appears to be conserved as it is also found in yeast [33]. These findings provide further evidence for K protein involvement in the dynamics of chromatin remodeling.

Newly synthesized transcripts are packaged into heterogeneous populations of nuclear ribonucleoprotein particles [34, 35]. K protein is one of at least 20 major proteins that compose the hnRNP particles [36]. Previous studies have demonstrated multiple direct interaction of K with other hnRNPs [32, 37]. Consistent with those prior studies we also isolated several hnRNP proteins that compose ribonucleoprotein complexes, including hnRNP A1, A2/B1, G, L, D, and U. The association with either hnRNP L or U that we observed here is likely to be direct since the same interactions were previously identified in yeast two-hybrid screens using K protein as a bait [32].

4.2 Cytoplasm

K protein's role in translations is well established [38–41]. Translation of 15-lipoxygenase (LOX) gene has been studied in most detail. Here, along with hnRNP E1/2, K protein regulates the recruitment of 60S ribosomal subunit to form competent 80S ribosome that commences translation of LOX mRNA [38–40]. The eukaryotic ribosome consists of four ribosomal RNAs and approximately 80 highly conserved ribosomal proteins [42–44]. We have identified several ribosomal proteins, which interacted with K protein. Three of them, acidic ribosomal proteins P0 (Arbp), P1A (Rplp1), and P2B (Rplp2) which form a pentameric complex bound to 26S rRNA [45], interacted with K protein *via* RNA. The other associations with ribosomal proteins appeared to be inde-

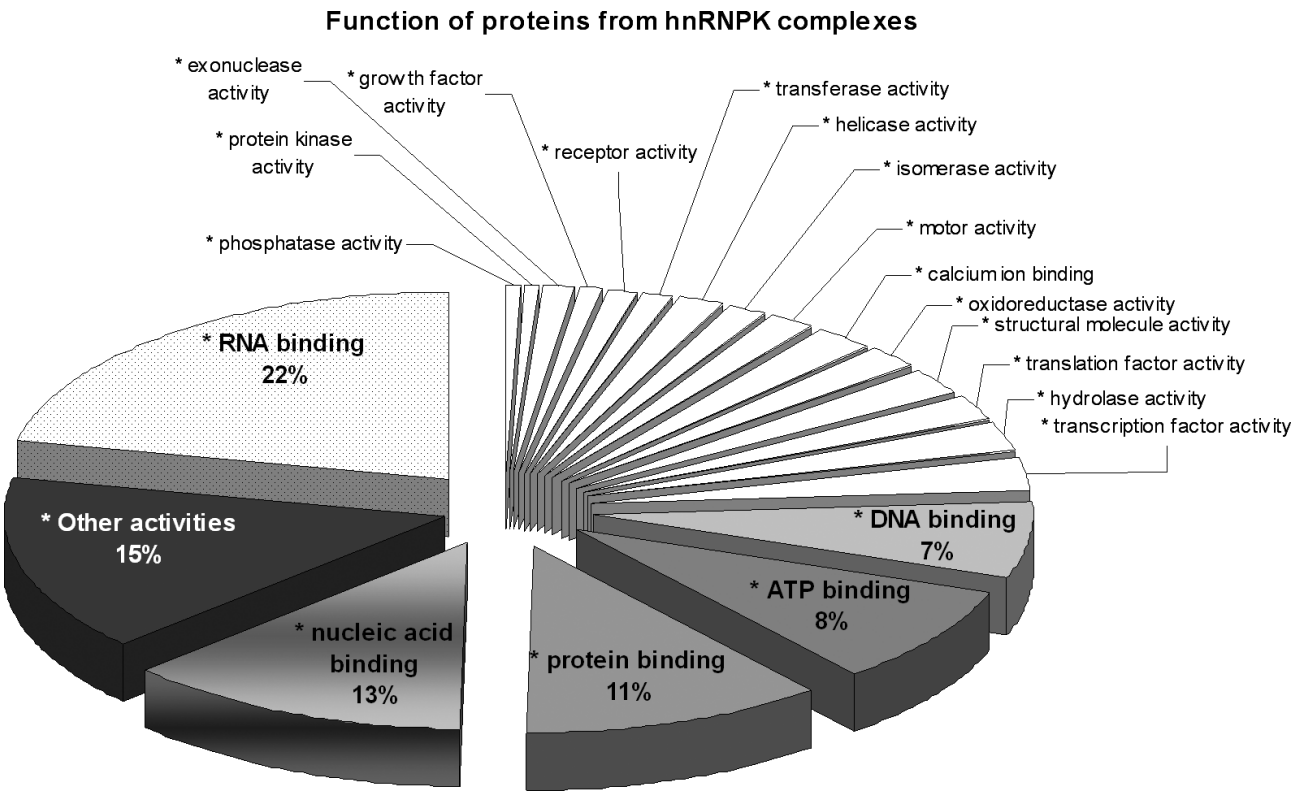

Figure 5. Diagram presents proteins interacting with hnRNP K proteins that were divided accordingly to their cellular function as determined by Gene Ontology.

pendent of RNA. *Caenorhabditis elegans* K protein encoded by the F26B1.2 [1] interacts directly with F13b10.2, an ortholog of mammal ribosomal protein Rlp3 (Table 1). Thus, the interaction of K with ribosomal proteins appears to be conserved.

Both our studies and those performed by Yano *et al.* [46] also revealed the interaction between K protein and the RNA-binding protein, Hu (Elavl1). Hu together with K protein regulates p21 mRNA translation [46].

C1qbp (p32) was one of the most frequently found factor in hnRNP K protein-associated complexes. C1qbp is involved in regulation of pre-mRNA splicing, mitochondrial transport, and oxidative phosphorylation, and in viral processes [47–49]. C1qbp interacts with several viral proteins, including EBNA I protein from Epstein-Barr virus [50, 51], two proteins Rev and Tat from human immunodeficiency virus (HIV) [52, 53], and IE63 (ICP27) from herpes simplex virus type 1 (HSV-1) [47] (Fig. 4). K protein interacts not only with IE63 (ICP27) [47] but also with several other viral proteins [47, 54–56] and regulates viral replication [57].

We found that K protein also interacts with several other proteins involved in RNA biology, including RNA helicases (Ddx1, Ddx5, and Ddx17). hnRNP K-RNA helicase interactions are consistent with the previously described association with DDX1 [58]. Micro-RNAs (miRNA) are increasingly being recognized as major regulators of gene expression [59]. Gregory *et al.*[60], reported that Drosha complex, involved in processing of miRNA, contains 20 proteins that besides several hnRNPs includes helicases (DDX1, DDX5, and DDX17) and proteins with dsRNA-binding activities. Based on these observations it seems plausible that the K protein–helicase interaction could play a role in the biogenesis of miRNAs.

The newly described SICs composed of ribonucleoprotein complexes take part in early stages of cell migration [22]. These ribonucleoprotein complexes are distinct from focal adhesions and implicate for the first time RNA and RNA-binding proteins including hnRNP K in the initiation of cell spreading [22]. We identified several cytoskeletal proteins that appear to form complexes with K. Some of these newly identified interactions may play a role in the early stages of cell migration.

4.3 Mitochondria

Fifteen proteins found here that were complexed with K protein have known mitochondrial location. These included outer (Raf1, protein serine/threonine kinase) and inner membrane proteins (adenine nucleotide translocators, mitochondrial F1 complex delta subunit proteins, and components of cytochrome b-c1). There were also several mitoplasm factors including heat shock proteins.

Table 2. List of hnRNP K interacting proteins and their orthologs divided by functional categories

	Molecular function/ cellular localization	Yeast	Worm	Fly	Mammals
1	RNA binding/ processing	YML117W Yra1, Sec65	T21G5.5 Y59A8B.10 C25A1.4 Y48B6A.3	Pasilla	Hnrpa1, Hnrpa2b1 Hnrpg, Hnrph Hnrpl, Hnrpd, Hnrpu, Ddx1,Ddx5, Ddx17, Elavl1, Sfpq, Eef1a1, Poldip3, Nsep1, Xrn2, Pairbp1, Eno1, Taf15, Naca, SRp20, RBM3, SAFB, Sam68, 9G8, hnRNP E2, hnRNP I
2	Rybosomalproteins/ translation factors	F13b10.2 Yef3, Cdc33, Sui3, Tif4631, Tif4632,Tif4632	–	–	Rpl23a, Rpl9, Rps18, Rps3, Rps5, Rps4, Rps25, Rps19, Rps2, Rps28, Rps14, Rps20, Rps23, Rpl28, Rpl31, Rpl30, Rpl22, Rpl11, Rpl13, Rps3a, Rpl23, Rpl37a, Lamr1,Rplp2, Rplp1, Arbp Eef1a1, Nsep1 (YB-1), Eif3s5, Eif3s6
3	Chromatin Processing	Hmo1, Hhf2	–	–	H2a, H2b, H3, H1d, DNA-methyltransferase, EED
4	Mitochondrial proteins	Mis1, Mrp7	C17h12.14	–	C1qbp, Atp5b, Atp5a1, Hspa9a, Hspd1, Acadl, UPla, Acat1, Prdx3, Aldh2, Uqcrfs1, Uqcrc2
5	DNA binding	Stm1, Ddr48	Y48B6A.3	Hey	Nsep1 (YB-1), Eef1a1, Hnrpu, Sfpq, Ddb1, Eno1
6	Transcription factors	–	Y49E10.14	Hey	Nsep1 (YB-1), Eef1a1,SAFB, Sam68, Zik1, Kid1, MZF-1, TBP, HMBG1, Purα, Sox10, C/EBPβ
7	Protein binding	–	F59E12.4, F13B10.2	–	Ribosomal proteins,Hspa8, Hspa9b, Hspa9a, Hspd1, Cct5, Hspa2, Actb, Actn4, Lmna, Actg1Çapza2, Grb2, Anxa2, Gfap, Krt2–6a, Mif, Chrdl2, Talin, Vinculin, Sam68
8	Protein kinase	–	–	–	Raf1, Itk, p85, Fyn, Lyn, Scr, PKCδ, PKC, PKCα, ERK1/2
9	Signal transducers	–	–	–	Grb2, Vav
10	Other enzymes	–	–	–	PRMT1 Pfkl, Tpi1

Underlined – Mammalian proteins interacting with hnRNP K protein that were found by others, but not in these studies.

Although K protein is localized within the intracristal mitochondrial space (Fig. 4) [61], it does not contain amino acid sequence that resembles known mitochondrial localization signal [62, 63]. This raises a question of how K protein is transported through the outer and inner mitochondrial membranes. Analysis of the mitochondria proteome identified several members of the major chaperone families [64]. Among them, the mitochondrial matrix Hspa9A (mtHsp70) is essential for the translocation of cytosolic proteins across the two mitochondrial membranes [65]. Mitochondrial Hspd1 (mtHSP60) is another required chaperon in the organelle transmembrane protein transport [65, 66]. The interaction of K protein with mtHSP70 and mtHSP60 identified in the current study opens up a way to explore mitochondrial import of K protein.

4.4 Plasma membrane

K protein is involved in signal transduction pathways initiated by a host of ligands [7, 10, 67, 68] and changes in extracellular environment [19]. This function reflects K protein interaction with Src-family of kinases [6, 7, 19], the nucleotide exchange factor Vav [4, 7, 10], PKC [8, 19], Erk1/2 [9], and likely many other kinases and signal transducers. These interactions have previously been identified using Western blotting but not in the current study using MS analysis. The stoichiometry of these interactions is in the range of 5–10% [8, 19], which is sufficient to be detected by antibodies but not by MS.

A recent large-scale proteomic study identified complexes composed of proteins that are tyrosine phosphorylated in response to EGF and PDGF [11]. Among more than 100

proteins that were identified in that study hnRNP K was only one of two RNA-binding proteins. The adapter protein Grb2 plays a critical role in signaling by receptor tyrosine kinases not only for EGF and PDGF but also for insulin [69–71]. The interaction of K protein with Grb2 (Fig. 2) is consistent with the observation that K protein is tyrosine phosphorylated in response to several extracellular signals, including insulin [10, 67]. This interaction adds further evidence for the role of K protein in coupling signal transduction pathways to RNA-directed processes [10, 67], including cascades initiated by receptor tyrosine kinases.

4.5 hnRNP K protein–protein interactions

3-D protein structure has provided much insight about the specificity of enzymatic reactions. Identification of the ancient multifunctional highly interactive RNA/DNA-binding proteins such as hnRNP K [1, 2] or YB-1 [72] thus counters the classical view of biochemistry.

There are more than 100 known hnRNP K protein partners and many more are likely to be discovered (Table 2) [1, 2]. How could a protein that has only a few well-structured domains associate with so many partners? Although some of the associations identified in this and other studies may represent indirect binding, many of these interactions are direct. There are entire proteins that appear to lack 3-D structure [73]. It is becoming apparent that these intrinsically disordered protein regions are functional and, in fact, play key roles in signal transduction and gene expression [74]. K protein contains a region (215–383 aa) that lacks a well-structured 3-D fold [75]. Indeed this stretch, previously designated as the K protein interactive (KI) region [2], mediates many of the known K protein–protein interactions [1]. SH2 and SH3 interactions are structurally well understood [76]. For some of the K protein interactions, the KI region's SH2 and SH3 docking sites are sufficient to mediate specific binding [2, 7, 77]. But what about most of the other KI region-mediated interactions? Many unstructured protein regions fold on binding to their partners as in the case of the interaction of the CREB transcription factor with CBP through its disordered KIX domain [78]. It seems plausible that coupled folding and binding [45, 73, 74, 78] could be the underlying processes responsible for the interaction of the KI region with many of its targets. The KI region interactions are regulated by phosphorylation [8, 19], a feature that is commonly seen in the binding of intrinsically disordered proteins where the interactions with biological targets is regulated by covalent modification [73, 78]. Phosphorylation of these and other regions may explain the dynamic nature of K protein complexes (Table 1).

Large-scale interaction experiments in *Saccharomyces cerevisiae* [79], *Drosophila melanogaster* [80], *C. elegans* [81], and man [82, 83] showed that most proteins interact with one or two other proteins, while a few so-called hubs interact with a large number of factors. As a result, such systems can be modeled as networks with an apparent scale-free topology [84]. For example, mapping of the fly and man protein–protein interactome identified several protein that have 50–100 protein partners [80, 82, 83]. Identification of highly interactive proteins in these large-scale experiments [82, 83] is also consistent with the results of this (Fig. 5) and other K protein interaction studies [1]. Many of the classes of mammalian hnRNP K protein partners have also been identified in yeast, worm, and fly (Table 2). Phylogenetic analysis revealed that through evolution K protein gained new docking sites that can recruit additional kinases and other factors involved in gene expression and signal transduction [1]. This fact and the observation that some of the fly proteins interact with as many as 100 proteins would predict that the scope of the mammalian K protein–protein interactome landscape is even larger than the one presently composed (Fig. 5).

Systematic functional analysis of the hubs identified in the yeast [79], worm [80], flies [81], and man [82, 83] protein–protein networks has not been reported. K protein is composed of domains that on one hand recruit kinases, while on the other hand bind chromatin, transcription, splicing, and translation factors. Many of K protein associations with its molecular partners are regulated by phosphorylation rendering these complexes dynamic (Table 1). Prior observations support a model where K protein acts as a docking platform at sites of nucleic acid-directed processes where it integrates signals from signaling cascades [1, 2]. Identification of novel K protein partners in the current study opens up new avenue to test this model in processes that link signal transduction to gene expression from plasma membrane to the nucleus.

The current study identified a large repertoire of K protein partners found in plasma membrane, mitochondria, cytoplasm, and the nucleus. It remains to be tested if coupled folding and binding of unstructured regions, covalent modification and the scale-free topology of protein–protein networks account for the rich landscape of the K protein–protein interactome.

This work was supported by grants from the Polish Committee for Scientific Research (PBZ-KBN-039/PO4/2001) and from the Polish Pharmacy and Medicine Development Foundation. J. O. research is also supported by a SCHOLAR GRANT from the Foundation for Polish Science. L. S. W. is supported by a Program for Young Researchers from the Foundation for Polish Science. K. B. is supported by the NIH GM45134, DK45978, and the Juvenile Diabetes Research Foundation.

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