

## Identification of Proteins that Associate with CAK $\beta$ /PYK2

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### ABSTRACT

Soluble cellular proteins were pulled down together with CAK $\beta$  protein tagged with FLAG-epitope and expressed in 293 cells. These proteins were identified by peptide fingerprinting combined with protein identification by mass spectrometric analysis using a MALDI-TOF

mass spectrometer. Seventy hits were obtained by database search. KIAA0555-related gene product, RNA binding protein 6 (DEF-3), a polybromo-1 related protein, and huntingtin interacting protein 1 (HIP-1) were among the strong candidates.

**Key words :** CAK $\beta$ , Protein-protein interaction, MALDI-TOF mass

### INTRODUCTION

Cell adhesion kinase  $\beta$  (CAK $\beta$ , also called PYK2, RAFTK, and CadTK) is a focal adhesion kinase (FAK)-related protein tyrosine kinase. CAK $\beta$  and FAK share the same overall structure. Both FAK and CAK $\beta$  contain conserved sites within their C-terminal domains for binding the integrin-associated protein, paxillin<sup>1,2)</sup> and Hic-5<sup>3)</sup>. In spite of this, CAK $\beta$  does not strongly localize to focal adhesions in fibroblasts<sup>3,4)</sup>. In accordance with these observations, integrins are the major cell surface receptors for the activation of FAK but not of CAK $\beta$ . However, in some types of macrophages<sup>5)</sup> and osteoclasts<sup>6)</sup>, CAK $\beta$  is indeed the kinase that is activated by ligation of integrin. Furthermore, CAK $\beta$  N-terminal and kinase domains, when targeted to focal contact sites by the FAK-C-terminal domain in FAK<sup>-/-</sup> cells, can functionally substitute for FAK in rescuing the fibronectin-stimulated migratory and signaling

defects<sup>7)</sup>. These findings indicate that downstream effects of CAK $\beta$  activation may be similar to those of FAK activation. That is, FAK and CAK $\beta$  should be related to common signalings that are either provoked by cell attachment to the extracellular matrix or by stimulations that activate CAK $\beta$ , such as ligation to cellular receptors of growth factors, peptide hormones, antigens, and chemokines (reviewed by Sasaki T et al.<sup>8)</sup> and Avraham H et al.<sup>9)</sup>). However, the mechanisms and direct effects of CAK $\beta$  and FAK activation are still elusive.

One of the most effective approaches to elucidate the function of signaling proteins is to study their associated proteins. In order to identify CAK $\beta$ -associated proteins, a recombinant CAK $\beta$  tagged with FLAG epitope was expressed in 293 cells and was then pulled down with anti-FLAG antibodies conjugated to agarose beads. Proteins pulled down together with the expressed CAK $\beta$  were applied to SDS-

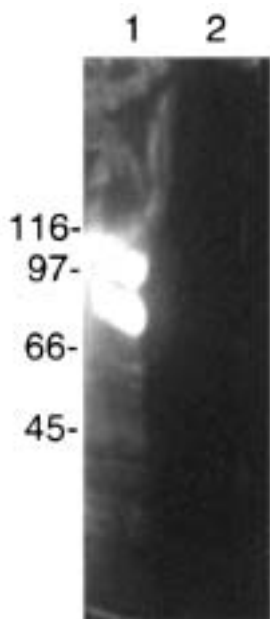
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PAGE and identified by a protein identification method combining in-gel tryptic digestion, MALDI-TOF mass analysis and a search of the database.

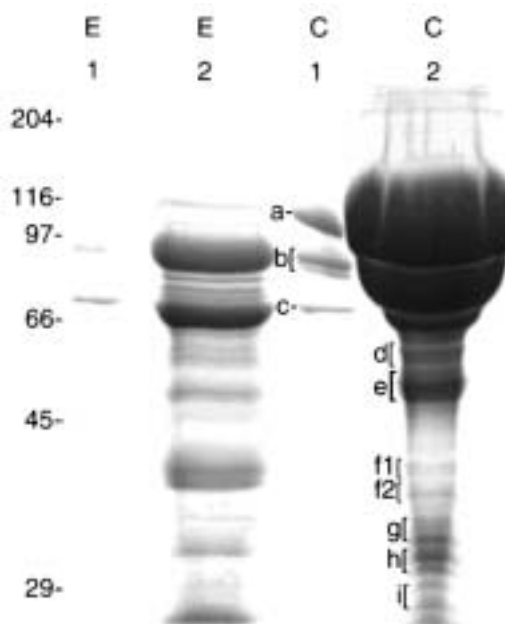
#### MATERIALS AND METHODS

Expression plasmids of the whole coding region of CAK $\beta$  and hEfs1 were constructed by insertion of BamHI-EcoRI fragment of GST-CAK $\beta$ <sup>3)</sup> and BstUI-StuI fragment of hEfs1<sup>10)</sup>, respectively, into BamHI site of pFLAG-CMV-5a by using a BamHI linker (New England Biolabs). Cells (20x10cm tissue culture dish of subconfluent 293 cells) were transfected either with FLAG-CAK $\beta$  or with FLAG-hEfs1, at 7 $\mu$ g of DNA per dish, by a Ca<sup>++</sup>-phosphate mediated DNA transfection method<sup>11)</sup>. FLAG-hEfs1 was used as a control to recognize nonspecifically bound proteins. Cells were collected 2 days after



**Fig. 1** Expression of FLAG-CAK $\beta$  protein in 293 cells. Subconfluent 293 cells were transfected either with FLAG-CAK $\beta$  (lane 1) or with FLAG-hEfs1 (lane 2). Cells were collected 2 days after transfection and lysed with lysis buffer. Lysate was centrifuged at 12000g for 10min and the resultant supernatant was mixed with 0.5 ml of anti-FLAG conjugated agarose beads (Sigma) at 4 °C overnight. Bound proteins were applied to SDS-PAGE with 10% acrylamide gel, transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon), and blotted with anti-CAK $\beta$  polyclonal antibody (Matsuya *et al.*, 1998).

transfection and washed twice with PBS. Cell packs were frozen at -80°C for up to a week. Cells were lysed with lysis buffer (0.1% Triton X-100, 150mM NaCl, 25mM Hepes pH7.2, 1mM EDTA, 50mM NaF, 1.5mM Na<sub>3</sub>VO<sub>4</sub> and protease inhibitors) and ultra-centrifuged at 30,000 x g for 20min. The supernatant was mixed with 0.5 ml of agarose beads conjugated with anti-FLAG M2 monoclonal antibodies (Sigma) at 4°C overnight, washed twice with TBS and the bound proteins were eluted with 1.2ml of 100  $\mu$ g/ml FLAG peptide (Sigma) in TBS. The eluted proteins were concentrated with centricon 50, washed with TBS, added to SDS sample buffer, heated at 60°C for 1hr and applied to SDS-PAGE with 10% acrylamide gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250, destained with 30% methanol and stored in deionized water until use. In-gel tryptic digestion and mass analysis were performed according by method used by Ishino *et al.*<sup>12)</sup>.



**Fig. 2** Samples prepared as in Methods were run on SDS-PAGE. After electrophoresis, gel was stained with Coomassie Brilliant Blue and then destained with 30% methanol. The name of each of the excised gel bands from which proteins were extracted for mass analysis are indicated. E:FLAG-Efs, C:FLAG-CAK $\beta$ , 1:one tenth of the sample, 2:the rest of the sample, M:molecular size marker (SDS-6H, Sigma)

**Table 1** Results from MS-Fit search.

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| DNA/RNA RELATED (23)                           |      |   |
|--|------|---|
| PID  | BAND | DESCRIPTION   |
| 6634015  | a    | RNA binding protein (KIAA0324)                                  |
| 15705403                                       | b    | proliferation potential-related protein PACT                    |
| 6634017  | b    | *KIAA0328 protein (Leucine zipper protein?)                     |
| 11968367                                       | c    | *novel KRAB box protein with 18 C2H2 type zinc finger domains   |
| 3334456  | c/f2 | *Activator 1 (RF-C) 140 kDa subunit (DNA-binding protein PO-GA) |
| 16878320                                       | e    | **Similar to KIAA0555 gene product (Leucine zipper protein?)    |
| 7448104  | f1   | gene MSH2 protein (mismatch repair protein)                     |
| 498152   | f1   | Zinc finger protein 33A (DNA binding protein)                   |
| 464325   | f1   | DNA replication licensing factor MCM3 (P102 PROTEIN) (P1-MCM3)  |
| 7573540  | f1   | RNA helicase  |
| 10436199                                       | f1   | Similar to Cip1/Waf1-interacting zinc finger protein ciz1       |
| 3702137  | f1   | dJ733D15.1 (Zinc-finger protein)                                |
| 1731434  | f1   | ZINC FINGER PROTEIN 41  |
| 16551459                                       | f1   | unnamed protein product   |
| 10432382                                       | f2   | novel protein similar to <i>Xenopus laevis</i> Sojo protein     |
| 16877622                                       | g    | protein for MGC:9466 = interferon-gamma induced protein         |
| 2995577  | h    | *unknown protein with AT hook (nuclear protein?)                |
| 12643357                                       | h    | **RNA binding protein 6 (DEF-3) (lung cancer antigen NY-LU-12)  |
| 14017821                                       | h    | KIAA1802 protein (Zinc finger protein)                          |
| 15126766                                       | h    | *RNA helicase   |
| 13469731                                       | h    | breast cancer antigen NY-BR-1.1 with bZIP site                  |
| 4826485  | i    | *TSBP (Testis Specific Basic Protein)                           |
| KINASE/PHOSPHATASE/G PROTEINS AND RELATED (11) |      |   |
| 3123587  | c    | cGMP-dependent protein kinase                                   |
| 2769645  | c    | ROS1 (transmembrane tyrosine-specific protein kinase)           |
| 15426510                                       | c    | *Unknown (protein for MGC:15356) = vav-like protein             |
| 16551971                                       | e    | **polybromo-1 related protein                                   |
| 12697995                                       | f1   | KIAA1725 protein (Dual specificity protein phosphatase?)        |
| 14133211                                       | f2   | KIAA0717 protein (Ras family ATP/GTP-binding protein)           |
| 12963885                                       | f2   | prostate antigen PARIS-1, with PH and TBC domains               |
| 1093486  | g    | *protein kinase C-related kinase:ISOTYPE=PRK1.1                 |
| 3043596  | g    | KIAA0536 = AY029347 serine/threonine-protein kinase (PRP4)      |
| 17380163                                       | i    | Dynamin-related 120 kDa GTPase (Optic atrophy 1 gene protein)   |
| 2209374  | i    | HsCdc7 protein kinase   |
| ENZYME/HOUSE-KEEPING (8)                       |      |   |
| 6760665  | b    | FLASH homolog RIP25 = caspase-8 associated protein 2            |
| 15277263                                       | b    | *alternative name: G2~unknown function (BAT2)                   |
| 13959398                                       | c    | serine protease inhibitor Kazal type 5                          |
| 7105924  | f1   | choline dehydrogenase   |
| 35053  | f2   | *uracil DNA glycosylase = glyceraldehyde-3-P dehydrogenase      |

17426470 h ubiquitin specific protease 9  
 3913330 h CYTOCHROME P450 2A13 (CYP11A13)  
 12698057 h KIAA1756 protein = rat CPG2 ortholog (carboxypeptidase G2)

## MEMBRANE/CYTOSKELETON/VESICLES (21)

2511666 a NrCAM protein  
 12642366 a \*myosin VI  
 190406 a profilaggrin (a major epidermal calcium-binding protein)  
 14211720 a desmuslin  
 1346640 b \*MYOSIN HEAVY CHAIN, NONMUSCLE TYPE B  
 15212240 b kinesin superfamily protein 1B  
 7529549 c \*novel protein similar to KIAA0884/NEUROFILAMENT TRIPLET M  
 15418997 c capillary morphogenesis protein-1  
 13431562 c \*\*HUNTINGTIN INTERACTING PROTEIN 1 (HIP-1) (cytoskeleton protein)  
 13397859 f1 KIAA1590 (novel protein similar to KIF1)  
 12697913 f1 KIAA1684 =SNIP-b related protein  
 2511779 f1 beta III spectrin  
 6330522 f1 KIAA1209 protein (PH domain protein)  
 6960319 f1 adaptor-related protein complex AP-4 epsilon subunit  
 17389307 f2 Similar to leucine rich repeat (in FLII) interacting protein 2  
 4416404 g nebulin  
 3478639 g \*delta-adaptin (clathrin coat adaptor subunit)  
 14285340 g Trabeculin-beta  
 10799514 g Trabeculin-beta like protein (similar to KIAA0728/KIAA0465)  
 7243270 h KIAA1436 protein (prostaglandin F2 receptor negative regulator)  
 3327146 h KIAA0666 protein (Formin Homology 2 Domain protein)

## EXTRACELLULAR (1)

3510536 f1 collagen type IX alpha I chain, short form

## UNKNOWN (6)

15147715 c dJ501N12.5.1 (novel protein (contains FLJ20048))  
 16307470 f1 Unknown (protein for MGC:5365)  
 16553925 f1 unnamed protein product (no related sequences)  
 12053255 g hypothetical protein (no related sequences)  
 3688350 g similar to hypothetical proteins S.pombe C22F3.14C  
 4240195 h KIAA0853 protein (function unknown)

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Protein hits that were obtained both from the control and CAK $\beta$  lanes were subtracted from the list. Protein ID number (Entrez) and its brief explanation is shown for each entry. Entries indicated with two asterisks are the most reliable hits, followed by those with one asterisk.

Acc. # 13431562. HUMAN. HUNTINGTIN INTERACTING PROTEIN 1 (HIP-1).  
8/80 matches (10%). 111634.8 Da, pI = 5.14.

| m/z submitted | MH <sup>+</sup> matched | Delta ppm | start | end | Peptide Sequence<br>(Click for Fragment Ions) |
|---------------|-------------------------|-----------|-------|-----|---|
| 1416.7378     | 1416.7308               | 4.9619    | 400   | 410 | (R)RQREDTEKAQR(S)                             |
| 1416.7378     | 1416.7586               | -14.6869  | 486   | 497 | (K)TQEQLVLES(L)K(Q)                           |
| 1448.7690     | 1448.7498               | 13.3029   | 823   | 835 | (K)NSRWTEGLISASK(A)                           |
| 1741.8896     | 1741.8833               | 3.6272    | 883   | 898 | (K)ADKDSPNLAQLQQASR(G)                        |
| 1814.9624     | 1814.9401               | 12.2988   | 431   | 445 | (K)EKYSELVQNHADLLR(K)                         |
| 2031.0565     | 2031.0471               | 4.6485    | 469   | 485 | (K)KELEDSELRISDQGQRK(T)                       |
| 2273.2010     | 2273.1672               | 14.8847   | 447   | 466 | (K)NAEVTKQVSMARQAQVDLER(E)                    |
| 2473.2531     | 2473.2357               | 7.0309    | 937   | 956 | (R)QEMDSQVRVLELENELQKER(Q)                    |
| 2530.3308     | 2530.3048               | 10.2779   | 447   | 468 | (K)NAEVTKQVSMARQAQVDLEREK(K)                  |

Acc. # 16878320. HOMO SAPIENS. (BC017354) Similar to KIAA0555 gene product .  
8/82 matches (9%). 94934.6 Da, pI = 5.88.

| m/z submitted | MH <sup>+</sup> matched | Delta ppm | start | end | Peptide Sequence<br>(Click for Fragment Ions) |
|---------------|-------------------------|-----------|-------|-----|---|
| 1473.7852     | 1473.7735               | 7.8972    | 318   | 329 | (R)ETEKQCKPLLER(N)                            |
| 1473.7852     | 1473.7913               | -4.1525   | 784   | 795 | (R)IRDLEDKTDIQR(K)                            |
| 1667.8332     | 1667.8175               | 9.3785    | 761   | 773 | (R)KSREYDCQILQER(M)                           |
| 1689.8649     | 1689.8482               | 9.9093    | 164   | 178 | (K)KQVDEALSNIQADK(I)                          |
| 1705.8501     | 1705.8761               | -15.2415  | 735   | 748 | (K)FGELLSEKQEE(L)R(T)                         |
| 1800.0119     | 1800.0100               | 1.0287    | 322   | 336 | (K)QCKPLLERNKCLAKR(N)                         |
| 1811.9756     | 1811.9867               | -6.1017   | 394   | 408 | (K)LQVIEQQNIIDELTR(D)                         |
| 2295.1142     | 2295.1012               | 5.6240    | 263   | 283 | (K)REIPGRAGDGSEHCSSPDLRR(N)                   |
| 2708.3870     | 2708.4154               | -10.4743  | 774   | 795 | (R)MELLQQAHRIRDLEDKTDIQR(K)                   |

Acc. # 12643357. HUMAN. RNA-BINDING PROTEIN 6 (DEF-3/NY-LU-12/G16)  
9/63 matches (14%). 128616.9 Da, pI = 5.93.

| m/z submitted | MH <sup>+</sup> matched | Delta ppm | start | end  | Peptide Sequence<br>(Click for Fragment Ions) |
|---------------|-------------------------|-----------|-------|------|---|
| 1167.6100     | 1167.6156               | -4.8031   | 841   | 850  | (K)SSSKKEMSKR(D)                              |
| 1294.6387     | 1294.6252               | 10.4265   | 209   | 218  | (R)EQSRSDFRNR(D)                              |
| 1422.6935     | 1422.6977               | -2.9738   | 859   | 871  | (R)GVTRFQENASEGK(A)                           |
| 1506.7088     | 1506.6924               | 10.9055   | 937   | 948  | (R)EEQTKKENEEDK(L)                            |
| 1650.8313     | 1650.8424               | -6.7741   | 1101  | 1113 | (K)RQSNETYRDAVRR(V)                           |
| 1662.8140     | 1662.7935               | 12.3619   | 936   | 948  | (K)REEQTKKENEEDK(L)                           |
| 1850.8795     | 1850.8745               | 2.6674    | 432   | 446  | (K)TARDAQRDLQDQDYR(T)                         |
| 2162.9852     | 2162.9678               | 8.0503    | 119   | 136  | (R)DIHSGDFRDREGPPMDYR(G)                      |
| 2295.0659     | 2295.0390               | 11.7283   | 239   | 258  | (R)GSGTTDLDFRDRDTPHSDFR(G)                    |

Acc. # 16551971. HOMO SAPIENS. (AK056541) unnamed protein product .  
8/82 matches (9%). 121647.3 Da, pI = 6.51.

| m/z submitted | MH <sup>+</sup> matched | Delta ppm | start | end  | Peptide Sequence<br>(Click for Fragment Ions) |
|---------------|-------------------------|-----------|-------|------|---|
| 1404.7201     | 1404.7348               | -10.4611  | 818   | 828  | (K)HLHNDVEKERK(E)                             |
| 1473.7852     | 1473.7742               | 7.4548    | 321   | 331  | (K)KKYPDYYQIQ(M)                              |
| 1582.7759     | 1582.7865               | -6.7216   | 164   | 177  | (K)NAKTYNEPGSQVFK(D)                          |
| 1805.8987     | 1805.8896               | 5.0353    | 758   | 771  | (R)LDLDFQEHMFVLER(A)                          |
| 2441.2586     | 2441.2676               | -3.7063   | 1009  | 1031 | (R)DVPLPVVRVASVFANADKGDDEK(N)                 |
| 2708.3870     | 2708.3758               | 4.1370    | 167   | 189  | (K)TYNEPGSQVFKDANSIKKIFYMK(K)                 |
| 2910.3201     | 2910.3654               | -15.5697  | 343   | 366  | (K)LKNQEYETLDHLECDLNLMFENAK(R)                |
| 2940.5233     | 2940.5583               | -11.9249  | 1005  | 1031 | (R)FVPRDVPVVRVASVFANADKGDDEK(N)               |

**Fig. 3** Detailed results of four hits selected from the MS-Fit search results. m/z submitted: mass data obtained from sample analyses, MH matched: theoretical mass data in the data base, Delta ppm: difference between submitted and calculated masses, start end: the amino acid numbers of the peptide sequence.

**Table 2** Proteins hits obtained both from the control and CAK $\beta$  lanes.

## DNA/RNA RELATED(13)

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|          |  |
|----------|--|
| 10434634 | nuclear receptor transcription cofactor like protein                 |
| 462325   | Heat shock 70 kDa protein 1 (HSP70.1) (HSP70-1/HSP70-2)              |
| 4240211  | KIAA0861 protein (DBL's big sister/MCF2 transforming sequence-like)  |
| 121059   | GC-rich sequence dna-binding factor (GCF) (TCF-9)                    |
| 14250918 | SMC5 protein (SMC:structural maintenance of chromosomes)             |
| 12643886 | ATP-dependent RNA helicase DDX20/DEAD-box protein 20/DP103/GEMIN3    |
| 13279176 | Similar to putative nuclear protein                                  |
| 6330893  | KIAA1254 protein (cell cycle progression restoration 8 protein CCP8) |
| 12006358 | Tara (similar to putative nuclear protein)                           |
| 4704204  | novel Mitosis-specific Chromosome Segregation protein SMC1-like      |
| 14250918 | SMC5 protein   |
| 1142657  | X2 box repressor (transcription factor REST like protein)            |
| 288565   | DNA topoisomerase II   |

## KINASE/PHOSPHATASE/G PROTEINS AND RELATED (5)

|          |   |
|----------|---|
| 13272526 | protein kinase NYD-SPK (serine/threonine kinase 31)           |
| 2281289  | receptor tyrosine phosphatase IA-2beta/X/pi/ICAAR/IAR-like    |
| 7243103  | KIAA1361 protein (STE20-like serine/threonine-protein kinase) |
| 4589542  | KIAA0949 protein (CITRON protein)                             |
| 179395   | breakpoint cluster region (bcr)                               |

## MEMBRANE/CYTOSKELETON (5)

|          |  |
|----------|--|
| 12644170 | Integrin alpha-6 precursor (VLA-6)                         |
| 6692822  | nebulette (a 107kD nebulin-like protein in cardiac muscle) |
| 2506774  | keratin 8, type II cytoskeletal                            |
| 7512516  | hypothetical protein DKFZp434A128.1 (similar to myosin)    |
| 105475   | myosin-binding protein C, skeletal muscle                  |

## EXTRACELLULAR (2)

|         |                            |
|---------|----------------------------|
| 3242753 | carboxypeptidase           |
| 87169   | collagen alpha 2(VI) chain |

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## RESULTS

Expression of FLAG-tagged CAK $\beta$  protein was confirmed by western blot analysis (Fig.1). The larger one of the double bands recognized by anti-CAK $\beta$  antibody migrated close to the 116kDa marker and may have represented the whole molecule of CAK $\beta$ . The smaller band possibly represented degraded product. Migration profiles of proteins which bound either directly or indirectly to the FLAG-column and were eluted with buffer containing FLAG-peptide are shown in Fig.2. Although many common protein bands at about the same migration distances were seen between the lanes of CAK $\beta$  and control, there were also specific bands that were seen only in one of these lanes. Each of the prominent bands that were seen only in the CAK $\beta$  lane, and the corresponding part of the control lane, were excised from the gel and subjected to the mass analysis as described in Materials and Methods. The results from the analysis are shown in Table 1. Entries with more than eight peptide matches (as shown in Fig.3) are considered as "most reliable hits", and indicated with two asterisks. Entries with 6 or 7 peptide matches usually result, because, empirically, several non-specific hits with 6 or 7 peptide matches usually resulted from a single MS-Fit search. Only a few parts of these hits, which show a similar tendency in each of the discrepancies between matched peptide masses and theoretical values, are considered to represent the tendency of mass discrepancies in mass spectrometric analyses, and are indicated with one asterisk. Entries that were found in the data from both lanes could represent proteins that have bound nonspecifically to the column. These hits may also provide us with important information for further experiments of this kind and thus are shown in Table 2.

## DISCUSSION

The results obtained here should be carefully interpreted because not all of the entries obtained here may represent "true hits". Empirically, about half of the hits marked with one as-

terisk may represent proteins that were actually in the gel. However, even if the given hits are "true", at least some of them may still not be CAK $\beta$ -binding proteins, because the control hits obtained here may not cover all of the proteins bound to the column nonspecifically.

Nuclear proteins and membrane/cytoskeletal proteins were prominent among the hits obtained (Table 1). However, this may not directly reflect the localization of CAK $\beta$  within the cell, and may have resulted from the protocol applied for this experiment, because nuclear and membrane/cytoskeletal proteins are also prominent for non-specific hits (Table 2).

We selected four hits that are most likely, in terms of reliability of database search results, to have CAK $\beta$  binding properties. KIAA0555-related gene product, DEF-3 (RNA binding protein 6), a polybromo-1 related protein, and huntingtin interacting protein 1 (HIP-I) are among these proteins. Three of them are supposed to be proteins that bind to polynucleotides. KIAA0555-related gene product (GenBank accession No.AAH17354) has a sequence related to a basic region zipper mediating sequence-specific DNA-binding followed by a leucine zipper, shared by the bZIP superfamily of eukaryotic DNA-binding transcription factors<sup>13</sup>. DEF-3 is a lung cancer-related RNA binding protein<sup>14</sup>. Polybromo-1 is a protein with five bromodomains and a BAH domain (GenBank accession No.BAB71210). Bromodomain is found in a variety DNA-binding proteins and can interact with acetylated lysine<sup>15</sup>. It may be involved in protein-protein interactions and may play a role in assembly or activity of multi-component complexes involved in transcriptional activation. BAH domain, which is shared by proteins involved in DNA methylation, replication and transcriptional regulation<sup>15</sup>, may link these functions. HIP-I is expected to be the only cytoskeletal protein among the four. HIP-I binds specifically to the N-terminus of human huntingtin<sup>16</sup>. HIP-I has an Epsin N-terminal homology domain and I/L WEQ domain. Epsin N-terminal homology domain, a domain of unknown function, is

found in proteins involved in clathrin-mediated endocytosis and cytoskeletal machinery<sup>17,18)</sup>, while I/LWEQ domain is shown to bind to F-actin<sup>19)</sup>. Because CAK $\beta$  is a protein which is indicated to be linked to cytoskeletal reconstruction and also to specific gene expression, a clearer understanding of possible bindings of CAK $\beta$  to these four protein could be very important for elucidation of the role and effect of CAK $\beta$  activation.

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