Identification of a large Myc-binding protein that contains RCC1-like repeats

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ABSTRACT The protooncogene MYC plays an important role in the regulation of cellular proliferation, differentiation, and apoptosis and has been implicated in a variety of human tumors. MYC and the closely related MYCN encode highly conserved nuclear phosphoproteins (Myc and NMyc) that apparently function as transcription factors in the cell. We have identified a large and highly conserved nuclear protein that interacts directly with the transcriptional activating domain of Myc (designated "protein associated with Myc" or Pam). Pam contains an extended amino acid sequence with similarities to a protein known as regulator of chromosome condensation (RCC1), which may play a role in the function of chromatin. The gene encoding Pam (PAM) is expressed in all of the human tissue examined, but expression is exceptionally abundant in brain and thymus. Pam binds specifically to Myc, but not NMyc. The region in Myc required for binding to Pam includes a domain that is essential for the function of Myc and that is frequently mutated in Burkitt's lymphomas. PAM is located within a 300-kb region on chromosome 13q22.

The *MYC* protooncogene is the archetype for a small gene family that also includes *MYCN*, *MYCL*, *MYCB*, and *MYCS*. The members of this family share four conserved domains: the Myc homology boxes I and II, a highly acidic region, and a composite known as the basic region/helix-loop-helix/ leucine zipper domain (1–3). *MYC* has been implicated in the control of normal cellular proliferation, differentiation, and apoptosis (1–3). In addition, diverse forms of evidence indicate that *MYC* can contribute to tumorigenesis. Aberrant expression of the gene has been found in many human tumors, overexpression of the gene can act either alone or with the activated *H-RAS* oncogene to transform cells, and certain transgenes of *MYC* are tumorigenic (1–3).

Previous studies have suggested that the product of MYC (Myc) may function as a transcription factor (1–3). The N-terminal domain containing the Myc homology boxes is essential to transcriptional activation by Myc (1–3). The C-terminal basic region/helix-loop-helix/leucine zipper domain mediates dimerization of Myc with other proteins and binding to a specific site in DNA (1–3). Myc also interacts with a wide variety of other proteins. Some of these interactions have well-established functional consequences, others do not. For example, dimerization with a protein known as Max is essential for the binding of Myc to DNA (4). In addition, the C-terminal domain of Myc binds the transcription factors TFII-I, YY1, and AP-2 (5–7).

The N-terminal domain of Myc also interacts with several proteins. These include the tumor suppressor protein p107, which represses transcriptional activation by Myc (8); Bin1,

another tumor suppressor protein, which binds to Myc box I and inhibits transformation by MYC (9), the TATA-box binding protein TBP (10); mitogen-activated protein kinase (11); and α -tubulin (12).

We now report the identification and characterization of a large nuclear protein that interacts with the transcriptionalactivating domain of Myc ("protein associated with Myc" or Pam). The gene encoding Pam (*PAM*) is contained within a 300-kb domain at chromosome 13q22, which also carries a recently identified gene *CLN5* responsible for a variant form of late infantile neuronal ceroid lipofuscinosis (13).

MATERIALS AND METHODS

Cell Cultures. Normal human aortic endothelial cells (HAEC) were from Clonetics (San Diego) and cultured in endothelial cell growth medium (EGM) (Clonetics) with 2% fetal bovine serum. CB33-Myc cell is an Epstein–Barr virus-transformed lymphoblastoid cell line overexpressing Myc from a transfected *MYC* gene (8). Tissue culture cell lines Hela S3, JAR, and JEG-3 were from the American Type Culture Collection.

Construction of Plasmids. The *Eco*RI–*Ava*I insert of the plasmid pSP65-cMyc (14) was cloned into the *Ava*I site of pGEX-2TK vector (Pharmacia) to make the plasmid pGST-Myc1. An *Eco*RI linker was inserted into the *Xho*I site at the 5' end of *MYCN* in the plasmid pcN64RX (15), and the insert then was cloned into the *Eco*RI site of pGEX-3X vector (Pharmacia) to make the plasmid p3X-NMyc. The insert of p3X-NMyc was cut out with *Bam*HI and cloned into the *Sma*I site of pGEX-2TK vector (Pharmacia) to make the plasmid pGST-NMyc1.

To create a series of C-terminal truncation mutants of Myc protein, the plasmid pGST-Myc1 was linearized with *Sac*II and treated with *Bal*31Slow (IBI/Kodak). The *Bal*31Slow-treated plasmids were ligated, transformed into bacteria, and examined by restriction digests and sequencing. Plasmids pGST-Myc5–10 constructed were selected for protein expression in bacteria. Plasmid pGST-Myc3 was made by cloning the *Eco*RI–*Tth*3I fragment of pSP65-cMyc plasmid into the *Ava*I site of pGEX-2TK. Plasmids pGST-Myc2, 4, and 11–13 were cloned in pGEX-2TK by PCR with Vent DNA Polymerase (NEB, Beverly, MA).

Cloning of Human *PAM* **cDNA.** The glutathione *S*-transferase (GST)-Myc1 and GST-NMyc1 fusion proteins were ³²P-labeled and used to screen a human λ gt11 cDNA expression library made from the Akata cell line as described

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Abbreviations: GST, glutathione S-transferase; PAC, P1-derived artificial chromosome; PAM, protein associated with Myc; RCC1, regulator of chromosome condensation; YAC, yeast artificial chromosome.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF075587).

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FIG. 1. Pam binds specifically to Myc but not NMyc. Lyso-PAM0 and Lyso-PAM0' represent two lysogens isolated from the positive phage λ gt11- PAM0 by using the host bacterial strain Y1089r-. Equal amounts of extracts from Lyso-PAM0, Lyso-PAM0' or host bacteria Y1089r- were fractionated by electrophoresis through a PAGE gel. The proteins then were transferred to a nitrocellulose filter, and the filter was incubated with various probes.

(16). The lysogens were isolated by using λ gt11-PAM0 phage and Escherichia coli starin Y1089r-, and the cell lysate was prepared as described (17). The conditions for the Far-Western hybridization were the same as those used for the original screening.

Human cDNA libraries made from Akata cells, T cells, testis, Raji cells, placenta, or brain were screened sequentially with the 5'-most or 3'-most cDNA fragments of the identified portion of PAM gene. After 13 rounds of screening, 15 kb of cDNA sequence was obtained and sequenced in both orientations.

The database searches and motif identifications were carried out by using the BLAST program. The sequence comparison and multiple-sequence alignment were carried out by using the programs of the Wisconsin Package Version 9.0, Genetics Computer Group (Madison, WI).

Immunofluorescence Staining and Immunoprecipitations. The protein GST-Ab1 containing residues 4312-4641 of Pam was expressed in bacteria and used to raise antibodies in rabbits. The antisera were depleted with GST and then affinity-purified by using GST-Ab1 on glutathione agrose beads. Cells were fixed in cold methanol and permeablized in 0.1%Triton X-100. The secondary antibody was the Cy3-conjugated goat antibody against rabbit IgG.

For immunoprecipatation, 1 ml of Hela nuclear extract (6 mg/ml, from Upstate Biotechnology) was incubated with 50 μ g of random rabbit IgG and 100 μ l of Protein A/G PLUS-Agrose beads (Santa Cruz Biotechnology) at 4°C for 2 hr. After the IgG bound to the beads was removed, 200 μ l of the depleted Hela nuclear extract aliquots was incubated with the primary antibodies Ab1 (anti-Pam), anti-c-Myc (Upstate Biotechnology), cv3 (anti-c-Myc), or random IgG. After 20 µl of Protein A/G agrose was added to the nuclear extract and incubated for another hour at 4°C, the agarose beads were washed with cold PBS four times. The immunoprecipitated proteins were fractionated on a PAGE gel and analyzed by Western blot.

P1-Derived Artificial Chromosome (PAC) and Yeast Artificial Chromosome (YAC) Clones. YAC clones containing the genomic PAM gene were selected by screening the whole genome YAC library (18) by using oligos in PAM gene CCTACTGTGGGAATCTGACCTC and CTGCCCGT-GAAGAGGCGGGCATG. The chromosomal locations for the YACs identified were obtained via the human genome web site (http://www-genome.wi.mit.edu). PAC clones were ob-

1	MPVPDGSVAA	AGLGLGLPAA	DSPGHYOLLL	SGRALADRYR	RIYTAALNDR	DOGGGSAGHP	ASRNKKILNK	KKLKRKOKSK	SKVKTRSKSE	NLENTVIIPD	IKLHSNPSAF	NIYCNVRHCV	
121	LEWOKKEISL	AAASKNSVOS	GESDSDEEEE	SKEPPIKLPK	IIEVGLCEVF	ELIKETRESH	PSLCLRSLOA	LLNVLOGOOP	EVLOSEPPEV	LESLFOLLLE	ITVRSTGMND	STGOSLTALS	
241	CACLESLVAS	WGETGETLOA	ISAILTNNGS	HACOTTOVET	ILNSLORSVO	AVEVGSTOTO	DWESNGTKKA	ALMHKWPLKE	ISVDEDDOCL	LONDGEFLYL	LCKDGLYKIG	SGYSGTVRGH	
361	TYNSTSRIRN	RKEKKSWLGY	AOGYLLYRDV	NNHSMTATRT	SPETLEODGT	VMLPDCHTEG	ONTLETDGEY	INOTAASROD	GEVVETEATS	TEPVLOOELO	LKLARKCLHA	CRISLEDLEK	LZ1
481	DLHIISTGFD	EESAILGAGR	EFALMKTANG	KIYYTGKYOS	LGIKOGGPSA	GKWVELPITK	SPKIVHESVG	HDGSHALLVA	EDGSIFFTGS	ASKGEDGESI	KSRROSKPYK	PKKIIKMEGR	adaga manazar (co.)
601	IVVYTACNNG	SSSVISKDGE	LYMEGRDATY	SDSSSLVTDL	KGHEVTOVAM	GKAHTCVLMK	NGEWWTFGVN	NEGOCORDIG	AMNOGGKGFG	VENMATAMDE	DLEEELDEKD	EKSMMCPPGM	RHD-1
721	HEWKLEOCMV	CTVCGDCTGY	GASCVSSGRP	DRVPGGTCGC	GSGESGCAVC	GCCKACABEL	DGOEAROBGT	LDAVKEMTPL	DELLAVPVPG	VNTEEHLOLE	OFEKRORVIE	RHRLEEGRGP	BR
841	LVEAGPIEMN	HREOALARUR	SHPAHVKHKR	DEFENDESCER	GENDASKITT	YPPGSVRFDC	FURAVOVSCO	EHHSVVLMEN	GDVYTEGYGO	HGOLGHGDVN	SRGCPTLVOA	LEGESTOVTA	RHD-2
961	GSNHTAVLLM	DOOVETEGSE	SKGOLGREIL	DVEYWNAKEA	PMPNTGSKYG	REATWIGASO	DOTFLETDEA	LINSHULATS	ETFASKHLIG	LVPASISEPP	PERCLATINKY	DGSCKTENDS	CDSM
1081	EOEDLOGEGV	CLOPVYDVTW	BERENTRELW	CYNAVVADAR	LPSAADMOSE	CSTLSPELAL	PTOSBALTTR	SHAALHILGC	LDTLAAMODL	KMGVASTEEE	TOAVMKVYSK	EDYSVVNREE	01.01.1
1201	SHGGGWGYSA	HSVEATRESA	DTDTLLGGLG	LEGGRGEYTA	KIKLEELGPD	GGDHETDGDL	LAETDVLAVD	CAAREKYAMM	EDEEVLLOAG	WWYVAWARVS	GRSSDCCSHG	OASTTTDDGV	PRI
1321	VEOEKSSKKS	NNGTOWNAGO	TPOLLYRLET	SDGSASKGKO	OTSEPVHILK	RSFARTUSVE	CFESLISTLY	MSWTTLVLGV	EELEGLEGEO	FTATLLDLER	LREVGTOCLE	LLEVYTCELY	
1441	PUSATGKAVU	RETSKLARCT	GKTETLLEKI	LSEPLDHCMV	KLONDROGYL	SOPLSLLEAV	LOECHNTETA	CENSEVETRA	LOWACLCDLL	NCLOODTOEA	NEKTSSSBLL	AAVMSALCHT	
1561	SVKUTSTEPT	AVDGEVLLES	TVKOVSTEND	STLVHREPLL	VAHMEKLSOS	FENISGMUSE	REVLERMENT	VULPURNSLR	RENELESSHL	VSNTCGLLAS	IVSELTASAL	GSEVDGLNSL	
1681	HSVKASANRE	TRUSOGREWN	TGNGSPDATC	ESVDXPGTVV	VGESVVGGGG	THEVELEVLY	DDSEHAGDST	HSHRWTSLEL	VEGUYTTODS	PSDTAFLRDD	KWVPLKENVK	VAVRLENYGS	PR2
1801	RTANGOGGMT	TWOCEDGV#Y	TESTOSLASN	GTNOTROUP	OLLYYRSEED	GDLOSOLLSK	ANEEDKNCSR	AL SWYSTWUR	ASKOLLHRAL	AVDADDTPRL	LSSSSLKSML	LPLIIAYIGP	1.1.2
1921	VAAATPKVAV	EVEGLYCOLL	PSVA ILNOKY	APPAFNENOS	TESTERNOPE	OCLSACTTSS	HVAVIESEND	VERACUMHYE	VTEPECVREM	TEEDBOCGT	AOSEDVERLE	LEVRIWONSG.	
2041	VGPKLTSVHE	NUNSWIELKK	FSGSSGWPTM	VLVLPGNEAL	ESLETASDVV	KDDKASEVGE	MORATOVERS	POPDEGVIOL	EKELANLGGV	CAAALMKKDL	ALPIGNELEE	DUETLERAAL	1.72
2161	OVCKTHSGIL	GKGLALSHSP	TILEALEGNI.	PLOTOSNEOS	FLODETACVP	GSSOGRLARM	LOPDSYADEO	KUSLILNKDD	TRCGMPTTTT	VOTKDOXGDV	VHVPNMKVEV	KAVPVSOKKM	N
2281	SLOODOAKKR	ORIPGSPANT	AASSNTDMEY	GGLASPKLDV	SVEPMIUKEA	RVIATTMMKV	VENVSEEELR	FASPTPERPS	ENMLTRVNND	GTYCANWTPG	A LOLYTIHVT	TOGTETDAGL	
2401	STONARDERG	MT DECIMALUK	DECEDUDANES	PREVAVOGAC	TETECUECTA	CENTOTORIN	CONTRACTORTO	MULTINAL DEM	DEBTERVION	MNCVPENUCT	CENCULORO	TUDUDESKTN	Max Di
2521	TODREKDING	COPORAMMOR	ODMEELBOOR	GMYRWARTGR	SCHNIRSCEN	TROTPTOMIA	LONKVKAVOR	VTNSECTION	LUONSMUREC	ESDEGEAWSE	ARDROONDYT.	RHEDEOALLD	Poni
2641	ONCOTIDDECD	FCUDAENKOA	CCANCEDVO	LONGROUPON	TOTOCEPACT	CONCRECCENT	COCT V DIVIDM	COLLINGTOCKED	DOMEST SAGE	CLITIKCDAAK	I Denenepet	CDMMMPLOP1	Kegn
2761	Venabwheed	DARCDORCOR	CCDEPERTO	ANDECDECAG	CDUCCCDURY	NI DOV CTA DU	VTVI DDDDVD	CKCHCAMAND	DULDSKKMDI	TWOIDCDCTC	DEDECTORNE	THURDOCEMDA	ano
2881	DODGING DOD	HCENNENCIEC	COLVENCEED	COUCDDOCERER	CUDRCCM204	FCTCKAPLED	EOEMDACDYT	CRECAMPNER	DISCRECCE	KODOSKPLED	AKONMEDEVA	SCADAVEACE	min
3001	Langervena	MACCOPIZER	DELCKENADI	DectMconbr	EFKERRY AND	HCIPTOCALM	MENTADIOR	1 CKMCC1NKN	KAUGWI KEDD	LERKCENCEP	ROUDELMONHN	THE CARACTERIE	
3121	TOHLWARKE	DTCI ATTEAA	CONNTEDEDC	SCAM AKKEC	FUCONVECCE	FREEKERAEN	UDDONL DODA	NOLAVCODER	DWICELCORG	HOVOUNVIND	OAHPCCORVA	GOODANGTON	NES
3241	RECOMMENCE	DIGLATINA	LUCDRODERV	LREKOAAARE	KUKOGDDKDM	OVERDDALDT	MENHOUTKAN	MURIL CL.CCA	AFDGTLCVHD	ANDEUGUI DG	WEGTCEDLD	VEMPOLATOR	112.03
3361	I NDHHHENEV	GYODDML FOD	EMEVIDENCY	DADVISUPDD	ACONVEREDE	COMPENDED.	FRODITION I	VKDUAEUDCA	SUMACEADRO	MOLLDYDANAY	TERESTSER	TEVOCELLEN	
3481	DODELODITO	VHGGI GAUED	NEOWDULAEV	TONNOLECLE	TAMKONLEKS	ACRUEAMEAE	NULCONTOR	TRUCTOFORDE	UNCIPENDUE	DEFERNMENT	TENCRORENCEOEK	DEPROCEHOLS	
3601	DIVIACEDAN	DI DEPENDI I	OPTEDI MARI	TOULDBOOD	I DOWSI KEKO	SDHOELHOSN	VENHIMMIC	Vennenerre	RETEINCORR	MSORICIUM	CLADINGTOD	TYPECDEAMT	
3721	COLADGENER	FWEGGDEDKN	VTENTTINCU	KGINYDAAGA	HUDNERDLCN	NAMENDEL DO	VAVEDICETY	OUDIDERATO	MARCEL BOOD	NHITKIRIKO	DEMTERNAN	KVI CWKDORG	
39/1	TRINCOLENC	VACORNCEAE	T DUPDITTC	OVECKLISCD	AFDWDROFFWW	ALLCODORE	VUVNAGEDAD	LANDORUTE	COCKLONI OK	OVCANIVOAT	DWEAMDODSE	WEWATCOVEN	
3061	AMCODNDEDA	CONVOURTI	CMULATOCOM	VCSOAL YOOL	THE ON POLICE	NEDGOT EGEL	UPCIIDDUID	ENERGYGITE	TOWNELDDYD	TOPLITOCAL	COMMERCELO	MELCOLARM	
4091	TRADE VANCET	THORN CHERLY	KCOMMUNIT DM	TENCOVIDEC	COMMING COM	DIVISION	I TROMANCHI	CENSCOUTEN	NTAEPTTALP	VMCCCCCCCDU	DOTATION	ALASI CULDO	
4201	DUDDICCOD	MCKDOOOKO	MDMCDNHDDC	ETA ATTICAT	CONLOTEOR	FIGUSICITIC	LODOVEVEEE	EXTRUDINEC	CODIVIENIM	ALADONUMEA	MUPPOPUTCE	DEMOCOLDO	
43.01	DUADRUSSOR	MAGNINGQQKQ	DCORVAGING	CUMUNOCUNO CUMUNOCUNO	CONTENDEDC	DOLACOVEN	TRUCK VENERE	CHICEMENT	AXIATOTROC	NUNDANIPINA	MULTING CON	TREPORTOGRE	DZE
4341	ONNEINHIU	KDULDDINEL	VEDIDERIAC	DI DVDCI UNC	PATERDONDE	VNDDACVAMI	TSHKUDADDA	OFFICE LEALS	CDARACDODD	ADDUBT LOCY	CONTENTATION	TILOLISCET	ICC P
4661	CONCERNING	RECECTORINE	NACUDDRODM	THE FREE PLAN	CDACDVCVOL	ECTECOL UNI	MINITY C. ERC.	CCCLCDNAR	E ACA1	LOLKEPICA	CODVORAQME	PARTURE	<u>Zn</u>
4001	N <u>URILLOVAV</u>	RHD-I	RHD-2	PRI	PR2	NGIECPENVV	Aye-Binding Region	HHD NI	.S			Zn	

FIG. 2. The structure and conservation of Pam. (A) Amino acid sequence of human Pam predicted from overlapping cDNA clones. The sequence includes the following motifs: LZ1 and LZ2, potential leucine zippers; RHD-1 and RHD-2, RCC1 homology domains; PR1 and PR2, Pam repeats; CDSM, cell division sequence motif; Myc-binding Region, insert in the original phage Agt11-PAM0; HHD, histone-binding protein homology domain; NLS, putative nuclear localization signal; RZF, potential ring zinc finger domain; and Zn, two putative C2H2-type zinc finger motifs. (B) Topography of human Pam. All abbreviations are as in A, except for SR, a serine-rich region occupying residues 2643–3057 (20% serine).

tained from the BacPac Resource Center at Roswell Park Cancer Institute, Buffalo, NY.

RESULTS

Identification of Pam by Protein Interaction Screening. To search for proteins that bind to the transcriptional-activating domains of Myc and NMyc, we fused N-terminal domains of the two proteins to the C terminus of GST, creating the plasmids pGST-Myc1 and pGST-NMyc1. The chimeric proteins were expressed in bacteria and ³²P-labeled as described in Materials and Methods. A mixture of the labeled proteins then was used to screen a $\lambda gt11$ cDNA expression library constructed from the RNA of the Akata Burkitt's lymphoma cell line. Two positive phages were identified. The positive phages were not binding to the GST portion of the probes (data not shown). Nucleotide sequencing revealed that these phages contain identical inserts of 902 bp, which encode an ORF of 300 aa. This ORF is fused to and in-frame with the LacZ protein encoded by the $\lambda gt11$ vector and contains neither a suitable start nor stop codon. Database searches failed to uncover any previous sightings of the ORF. We designated the encoded protein as Pam for "protein associated with Myc" and the phage containing the 902-bp insert as λ gt11-PAM0.

Pam Binds to Myc but Not NMyc. We explored the specificity of Pam binding in three ways. First, we demonstrated that plaques of λ gt11-PAM0 hybridized to GST-Myc1, but not to GST-NMyc1 (data not shown). Second, we removed the GST portion from the Myc and NMyc probes by cleavage with thrombin; the pattern of binding to λ gt11-PAM0 remained the same (data not shown). Third, we used GST-Myc1, GST-NMyc1, and the GST alone as probes in Far-Western blots (Fig. 1). Only the Myc probe bound to the LacZ fusion protein made from λ gt11-PAM0. We conclude that the interaction between Myc and Pam is sufficiently specific to exclude detectable binding to NMyc.

PAM Encodes a 510-kDa Protein with a Domain Related to Regulator of Chromosome Condensation, RCC1. To obtain a full-length cDNA representing the gene encoding Pam, we screened several cDNA libraries and isolated overlapping clones covering 15 kb of PAM cDNA. Sequencing of the overlapping cDNAs revealed an ORF of 4,641 aa with a predicted mass of 510 kDa (Fig. 2A). The encoded protein has several notable features (Fig. 2B): a ring zinc-finger motif (RZF) and two zinc-finger motifs (Zn); a bipartite nuclear localization signal (NLS); two putative leucine zipper motifs (LZ1 and LZ2); a cell division sequence motif (CDSM), found in a variety of viral and cellular proteins that have been implicated in control of the cell cycle (19); and a large region (residues 498-1065) exhibiting significant homology to a protein known as RCC1 (20). The original fragment of PAM accounting for 300 aa isolated by interaction screening encodes the middle of Pam; this fragment must contain a domain responsible for binding to Myc, but we have yet to localize that domain with any greater resolution.

The resemblance to RCC1 deserves further comment. RCC1 contains a motif of 50-60 aa that is repeated seven times in tandem. These repeats form a seven-bladed propeller structure as determined recently by x-ray crystallography (21). A similar 7-fold repeat is present in Pam, but is divided into two elements (RHD-1 and RHD-2) by an insertion of 134 aa after the fourth repeat (Fig. 2 and Fig. 3 *A* and *B*). The insertion between RHD-1 and RHD-2 contains a C-terminal region of 55 aa that is rich in basic amino acids (BR in Fig. 2*A*). Such a short basic region (40–50 aa) also is present in RCC1 proteins at their N termini, which are important for chromatin binding (20). Although most RCC1 proteins end with a repeated element, the *Drosophila* RCC1 protein BJ1 has a substantial C-terminal extension, which has limited homology to chromatin proteins such as *Xenopus* histone-binding protein

A		
RCC XENLA	50 GEMITVEL CHESTER	94
RCC MEMAN	75 GGMHTVCLSK3GOV	89
DOC NIDDA	105 2004 TAV THE 2004	119
RCC_ALRES		425
D01AKTD-1	4_1 @2017V131D#0VK	440
MBF-RHD-1	439 GREFALKSTRAKE	513
RCC_XENLA	TF@CNDECALG-RDTSEKGSEMOPGKVELAEKv@QVGA	147
RCC_HUMAN	SFGCNDEGADG-RDTSVEGSEMVFGKVELQEKVVQVGA	162
RCC_XLRP3	ATCONNEGOLGDTEERNTFHWISFTSEHKIKQLGASNTSAALTEDGRLEM	173
p619RLD-1	ACCKGSKGR OLGDSNNOSTLKK WITTEPHRS KKVSS SKGSDOHULAFTTEDEVRS	481
MBF-RHD-1	YTT - KROSLAUKOGOPSAGKWEEDPITKSPENNHPEVGHDG-SHADLVAEDIGIEF	567
RCC_XENLA	PROFEDENCYICLLEFMEKSMVPVQVQTWIPVIKTASGNDHLVLLIVDADAY	198
RCC_HUMAN	WEFRONNEV! CLEPNKKSNVPVQVQLEPNKKSNVPVQVQ	194
RCC_XLRP3	NGDNSEDOINLKNVG-NVCVECOVTIGEPVSWISEGYYNSAFVTTOGEN	222
p619RLD-1	NGD CDYCKLEHGNSK-TOKYEKLICGPLCCCVVVCVSAGYRHSAAVTEDEBL	533
MBF-RSD-1	TOSASKGEDGESIKSRROKSKEKKEIKMEGKINYTADMASSOVISKIGEL	622
RCC_XENLA	TSBCGEQGQLGRVPERFINEGRKGLERLLV-PQCIN L KAKGS-GRVHFQEMFCGAYFTFAVSQEWI-VY	266
RCC_HUMAN	TLECGECGOLGRVPELFANRGERGLERLLV-PXCVMLKSRGSRGEVRFQDAFCAAYFTFAISHEEH-AV	262
BCC XLRF3	VERPENSIK, GLENOLLGNIR	274
n619RLD=1	THE REPORT OF A DESCRIPTION OF A DESCRIP	584
MER-PUD-1	MICH AND A STATE AND A	663
NP5-XUD-1	NDCKOWII 20032	
BCC VENTA	います。 char una dire himohi	320
DCC HUMAN	CELEVILLING AND THE CONTRACT OF THE CONTRACT. OF THE CONTRACT OF THE CONTRACT. OF THE CONTRACT OF THE CONTRACT OF THE CONTRACT OF THE CONTRACT OF THE CONTRACT. OF THE CONTRAC	316
RCC_HORAN		100
RCC_XLRP3	APGLEGPEGDEL-GTPLPETERPRVI-RVIRDQUISL-SCENAR ANTICIDUDE	676
p619RLC-1	SPOGGDNGKLCHCDTNR	030
MBF-RHD-1	TRAVINGOCOR - DICHMOGGEGFGFGVENEADAMDED BEERDEEDERDER SEMCEPOMEERWEIGEGEG- Y	740
ъ		
D	the second se	1.00
ACC_XENLA	152 RINN-COTINLEPPERKSNVECCO	195
RCC_HUMAN	147 REMN-GVIGLLEPKEKSMVEVQVQDUVPVEKGASENDELQVQVA-SER	196
RCC_XLRP3	175 CDKSEQDIGEX-NVSNVCVB22VTIGKPVSWISCCYYNSAFVTTCELT	222
P619RLD-1	483 SECENCELOHG-NEETOKYEKIIQOPLQCKVVWCVSACYRESAAVTEDELIY	533
MBF-RHD-2	874 KOCS-GERGERDARKITTI YZPGSVRFDCELRAVCVSGGFHHSV/TD DORVA	924
RCC_XENLA	TSG GEOGLARVPERFINRGGR-KGMERLLVPQCIHLKAKGS-GRVHFQDVPCGAYF FAVSQBOH-WY	266
RCC_HUMAN	CCCCCCCCCCCRVFELFANRGGR-CCCERLLVPKCVMLKSRGSRGHVRFQDAFCGAYFFFAISHECH-WY	262
RCC XLRP3	VEGREVER AL PHOLLCHHRED - CALVEST PEKVIALACCENTRY	274
n619RUD-1	TWARE DEBT ARE DEMENDED TO VE DISN	584
MBF-RHD-2	TFOY GO, GODGRODVNSRCOPTLYQALPOP	975
RCC_XENLA	G₽ġLSNYHQQQCKN?QQCYA₽QO-₩LT₩FKNSTXSWQC%\$QQ0HHTVCVDQCSW4V	320
RCC HUMAN	GRELSNYHOLOY TO	316
SCC_XLESS	THE COTOMER, C	327
melant D-1	WEIVER WEIVER AND	636
DOLOKUD-1		103
MEP - RED-2	TEPD - OVACHARE'T	
RCC XENLA	SLORBEYGRIGISENABEQSEPTPIP 346	
BCC HUMAN	SCREENCELINGCECAEERSIECLIS 342	
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FIG. 3. Sequence alignments of the RCC1 homology domains (RHD-1 and RHD-2) and two additional direct repeats (PR1 and PR2). (A) Alignment of RHD-1 of Pam with RCC1 proteins. RC-C_XENLA and RCC_HUMAN are the RCC1 proteins from *Xenopus* and human (P25183 and P18754). RCC_XLRP3 is the X-linked retinitis pigmentosa 3 protein (O92834), which belongs to the RCC1 family. p619RLD-1 is the first RCC1-like domain in p619 protein (U50078). The underlining indicates the signature sequences of RCC1 family (PS00625 and PS00626). The arrow indicates the amino acid insertion of WKLEQCMVC. (B) Alignment of RHD-2 of Pam with RCC1 proteins. The arrow indicates the amino acid insertion of FLRI. All details are as in *A*. (C) Alignment of the two direct repeats of human Pam (PR1 and PR2).

N1/N2 (20). Pam contains a similar region (designated HHD, see Fig. 2*A*), situated in the midst of a serine-rich domain (SR) and in the vicinity of the Myc-binding domain, but relatively distant from RHD-1/2 (see Fig. 2*B*).

In addition to the RCC1 repeats, Pam contains two additional direct repeats of 91 aa (PR1 and PR2, Fig. 3*C*). These two repeats are 407 aa apart.

By searching several databases, we uncovered expressed sequence tags (ESTs) from the mouse and rat genomes that are represented within *PAM*, as well as a complete homologue of *PAM* in *Caenorhabditis elegans* (GenBank accession no. U53147). The sequences of human and *C. elegans* Pams display 32% identity and 55% similarity (data not shown). There are focal regions of exceptional conservation, but similarity is distributed throughout the whole ORF. The most conserved C-terminal region of 324 aa shows 55% identity and 73% similarity. Because the homologue in *C. elegans* was uncovered by genomic sequencing rather than genetic analysis, nothing is yet known of its function.

The rodent expressed sequence tags represent only portions of Pam, but are extremely conserved when compared with each other and to human Pam. The mouse ESTs cover the following residues in human Pam: 2575–2810 (90% identity, 93% similarity), 2912–3152 (94% identity, 97% similarity), 4061–4268 (99% identity, 100% similarity), and 4458–4641 (98% identity,

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FIG. 4. Pam RNA in human tissues. Northern blots prepared with RNAs from multiple human tissues were obtained from CLONTECH (MTN I and II). The filters were analyzed with radioactive probe prepared from *PAM* cDNA following the manufacturer's instructions.

99% similarity). The accession numbers for the mouse *PAM* ESTs are W44173, R75243, AA727659, AA689905, AA545634, AA547620, AA414366, AA734728, AA119999, AA647417, and AA174369.

PAM Is Expressed in Many Tissues but Most Abundantly in Brain and Thymus. We examined the expression of *PAM* by performing Northern blots with polyadenylated RNA from various human tissues (Fig. 4). A single RNA with a size of approximately 15 kb was detected in all tissues examined. The level of expression was relatively low in heart, placenta, lung, liver, kidney, spleen, prostate, testis, small intestine, colon, and peripheral blood leukocytes; somewhat higher in skeletal muscle, pancreas, and ovary; and highest in brain and thymus.

Pam Is Located in the Nucleus and Binds to Myc in Cellular Extracts. We prepared a polyclonal rabbit antiserum against the C terminus of Pam and purified the antiserum by affinity chromatography on the antigen. Immunostaining with the antiserum revealed strong nuclear fluorescence in interphase nuclei of normal human aortic endothelial cells (Fig. 5 A–C) and cultured cells of human choriocarcinomas (lines JAR and JEG-3; data not shown). In contrast, the fluorescence dispersed throughout the whole cell during mitosis, when the nuclear envelope is disintegrated (Fig. 5 D–F for a telophase cell).

Knowing that Pam is located in the nucleus during interphase, we examined the interaction between Pam and Myc in nuclear extracts. We used two experimental tactics. First, Myc protein in extracts could be bound to GST-Pam0 and then



FIG. 5. Subcellular localization of Pam. Immunofluorescence microscopy was performed on normal human aortic endothelial cells with antibody directed against Pam. (A) and (D) Immunofluorescence with Pam-specific antiserum. (B) and (E) Staining with 4',6-diamidino-2-phenylindole (DAPI). (C) and (F) Phase contrast microscopy. The bars represent 40 μ m in C and 20 μ m in F.



FIG. 6. Myc interacts with Pam in vivo. (A) Binding of Myc to GST-Pam0. GST and GST-Pam0 proteins were incubated with a nuclear extract of CB33-Myc cells, recovered on glutathione agarose beads, and analyzed by Western blotting by using antibody directed against Myc (9E10). Lane 1, binding with GST-Pam0 protein. Lane 2, binding with GST protein. Lane 3, total nuclear extract of CB33-Myc cells. (B) Binding of Pam to GST-Myc1. Purified GST or GST-Myc1 protein was incubated with a nuclear extract of Hela cells, recovered on glutathione agarose beads, and analyzed by Western blotting with an antiserum against Pam. Lane 1, binding with GST-Myc1 protein. Lane 2, binding with GST protein. Lane 3, the supernatant after adsorption of the nuclear extract with GST protein. Lane 4, the supernatant after adsorption of the nuclear extract with GST-Myc1 protein. (C) Coprecipitation of Pam and Myc from nuclear extracts of Hela cells. Extracts were subjected to immunoprecipitation with various antisera. The precipitates were analyzed by Western blotting with antiserum against Myc (9E10). Lane 1, total nuclear extract. Lanes 2-5, immunoprecipitates. Lanes 6-9, supernatants after immunoprecipitation. Lanes 2 and 6, immunoprecipitation with anti-Pam. Lanes 3 and 7, polyclonal anti-Myc from UBI. Lanes 4 and 8, polyclonal anti-Myc prepared by investigators (cv3). Lanes 5 and 9, nonspecific rabbit IgG.

recovered on beads coated with glutathione (Fig. 6A). Similarly, Pam could be recovered from nuclear extracts by incubation with GST-Myc1 (Fig. 6B). No binding could be detected between GST itself and either Myc or Pam (Fig. 6A and B). Second, Myc coprecipitated with Pam when the latter was recovered by reaction with the specific antiserum described above (Fig. 6C). We were unable to perform the reciprocal experiment, possibly because of the low abundance of Pam.

Pam Binds to a Region of Myc that Is Important for Transactivation of Transcription and that Is Frequently Mutated in Burkitt's Lymphomas. To localize the regions in Myc important for binding to Pam, we constructed a series of truncations of Myc and then examined their binding to LacZ-Pam0 in Far-Western blots (Fig. 7). Representative data are illustrated in Fig. 7A and summarized in Fig. 7B. Short truncations from the N terminus, up to the border of the Mvc box I domain, did not disturb the binding of Pam (Myc11, 12). In contrast, those truncations that extended into the Myc box I domain eliminated binding to Pam (Myc13). Truncations extending from the C terminus to residue 154 did not affect binding to Pam (Myc1-6). C-terminal truncations beyond amino acid 154 (Myc7-9) reduced but did not eliminate the binding, whereas truncations from the C terminus to amino acid 75 (Myc10) abolished all detectable binding.

In aggregate, the data indicate that the portion of Myc between amino acids 44 and 107 is essential for binding to Pam (Fig. 7*B*). This region contains the highly conserved Myc homology box I. Residues 140-154 also contribute to maximal binding, because the extent of binding drops substantially when this portion of Myc is removed. The Pam-binding region in Myc is important for the transcriptional-activating activity of Myc (1–3) and is frequently mutated in Burkitt's and AIDS-associated lymphomas (2).

PAM Is Located at Chromosome 13q22. We mapped the chromosomal location of *PAM* by screening a human genomic YAC library. The four YAC clones identified through the screening all have been localized to chromosome 13q22-31



B Localizing the Pam-Binding Regions in Myc Protein



FIG. 7. Localization of the Pam-binding site within Myc. (*A*) Binding of 32 P-labeled truncations of GST-Myc to LacZ-Pam0. Binding was assayed by using the Far-Western assay. (*B*) Summary of results. The binding data for some of the truncation mutants listed here are not shown in *A*. The numbers indicate the amino acid residues in human Myc.

(854b5, 852g2, 885a10, and 946c1). This region contains the *CLN5* gene, which is defective in a variant form of the human disease late infantile neuronal ceroid lipofuscinoses (13). The YAC that harbors the *CLN5* locus is identical to YAC 852g2, which contains sequences of *PAM*. To further authenticate the location of *PAM*, we compared the restriction patterns of YAC 852g2 and human genomic DNA when they were probed with *PAM* cDNA. The patterns obtained with 852g2 YAC were the same as those obtained with human genomic DNA (data not shown).

The location of *CLN5* has been refined to a 300-kb region between markers AC224 and COLAC1, covered by several PAC clones (see Fig. 8; ref. 13). We explored whether *PAM* is localized to this 300-kb region by hybridizing *PAM* cDNA probes to the restriction fragments of these PACs. A 3' cDNA probe of *PAM* (3HP3) hybridized with the restriction fragments from PAC76n15, whereas a cDNA probe near the 5' end (T25) hybridized with the restriction fragments of PAC189o20 (data not shown). These results demonstrate that the transcription of *PAM* must proceed from right to left along the *CLN5* locus as displayed in Fig. 8.

Recently, PAC264j2 and PAC76n15 were sequenced by the Whitehead Institute Genome Center (Cambridge, MA) (Gen-Bank accession nos. AC001226 and AC000403). These two PACs overlap by 17 kb and cover a total of 199 kb of genomic



FIG. 8. Mapping the *PAM* gene to the chromosome 13q22 region between markers AC224 and COLAC1. BTF3 gene and a pseudogene for RNA helicase A (DDX9P) were identified by random sequencing of PAC224a14 (22).

DNA. The AC224 marker is located within the PAC264j2 clone (nucleotides 32152–32173), whereas the COLAC1 marker lies outside the PAC264j2/PAC76n15 region (see Fig. 8). Sequence comparison between *PAM* cDNA and the sequenced genomic DNA in this region confirmed that *PAM* is located in this region as displayed in Fig. 8. The 3-kb cDNA at the 3' end of the *PAM* gene lies in the 30-kb terminal region of PAC76n15. The rest of the *PAM* cDNA lies in the nonsequenced genomic region in PAC189o20 or beyond.

These analyses indicate that *PAM* is located on PAC76n15 and PAC189020 between the markers AC224 and COLAC1 at the *CLN5* locus. To the best of our knowledge, however, *PAM* is not equivalent to *CLN5*.

DISCUSSION

We have identified a large nuclear protein, Pam, that interacts with a functionally important region of the protein encoded by the *MYC* protooncogene. *PAM* apparently is expressed in all of the tissues we examined, but expression is especially abundant in brain and thymus. *PAM* is located within a 300-kb domain at chromosome 13q22, in the vicinity of the recently identified *CLN5* gene (13).

Pam Binds to a Functionally Important Region of Myc. We have localized the site of Pam binding to a 64-aa region within Myc that includes the highly conserved Myc homology box I. The Myc homology box I is essential but not sufficient for Pam binding: the C-terminal truncation Myc10 leaves the box intact, yet abolishes the binding (see Fig. 7). For maximal binding, an additional region between amino acids 107 and 154 is required. This sequence might be essential for the correct folding of Myc or provide a second contact with Pam.

There is circumstantial evidence that the binding of Pam to Myc may be functionally significant. First, the binding occurs to a region of Myc that is essential for transcriptional activation by Myc. Second, point mutations cluster in this region of the translocated alleles of Myc found in Burkitt's and AIDSassociated lymphomas (2). These mutations are thought to augment the tumorigenicity of the translocated gene and bespeak a functional role for the affected regions of Myc (2). We hypothesize that the binding of Pam plays a role in transcriptional activation by Myc, either as facilitator or regulator.

Pam Binds to Myc but Not NMyc. Myc and NMyc are closely related proteins that share a number of biochemical functions, including binding to the same specific site in DNA (1–3, 23) and dimerization with the Max protein (4, 24). Both Myc and NMyc can induce S-phase DNA synthesis in established lines of rodent fibroblasts (25, 26), elicit extended proliferation of normal rodent fibroblasts (1, 27), transform Rat-1 cells (14, 28), and cooperate with *H-RAS* to transform embryonic rat fibroblasts (1, 29).

There are reasons to believe, however, that Myc and NMyc have different biological functions *in vivo*. First, the pattern of

expression differs for the two genes in both embryos and adults (30-33). Second, aberrant expression of *MYC* is associated with a large number and variety of human tumors, whereas *MYCN* has been implicated in a much more limited set of tumors (1-3). Third, homozygous deficiencies of either *MYC* or *MYCN* in mice are embryonic lethals (33-35). Thus, *MYC* and *MYCN* are not functionally redundant.

The biological differences between *MYC* and *MYCN* could be solely because of the different patterns of expression. But the specificity of binding between Myc and Pam suggests that an additional explanation for functional differences between *MYC* and *MYCN* may emerge once the function of the binding is understood.

The Myc homology box I is virtually identical between Myc and NMyc (20 of 23 amino acids in box I are the same). Because a truncation of Myc that leaves the Myc box I intact cannot bind to Pam (see above), it is likely that the specificity of Pam binding is determined by regions outside of the box.

RCC1 Motifs May Provide a Clue to the Function of Pam. Pam is located in the nucleus during interphase of the cell cycle, but disperses throughout the whole cell during mitosis, when the nuclear envelope is disintegrated and the condensed chromatin is neither transcribed nor replicated. Several motifs in Pam, such as leucine zippers, zinc fingers, putative histonebinding protein homologous domains, and the RCC1 motifs, suggest that the function of Pam may involve DNA binding and chromatin. Among these features, the RCC1 motifs occupy the largest region in Pam (\approx 500 aa). RCC1 may have multiple functions, but these remain poorly documented (20). One unifying hypothesis is that RCC1 may serve to either evoke or detect conformational changes of chromatin during the cell cycle (20). As a transcriptional activator, Myc must interact with chromatin. Pam may play a role in that interaction. The presence of a well-conserved Pam in C. elegans offers the prospect for a genetic analysis of the protein's function.

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