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PRINCIPAL INVESTIGATOR: Frank J. Rauscher, Ph.D.

CONTRACTING ORGANIZATION: The Wistar Institute
Philadelphia, Pennsylvania 19104

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Frank J. Rauscher, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Wistar Institute
Philadelphia, Pennsylvania 19104

E-Mail : rauscher@wistar.upenn.edu

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I. Summary

In this research project, we had proposed to investigate the utility of the *Bin1* gene as a diagnostic and/or prognostic marker in prostate malignancy and to perform proof-of-principle tests of its ability to induce apoptosis in androgen-independent cancer cells. Bin1 encodes a set of adaptor proteins, the first of which was identified in our laboratory by virtue of its ability to interact with and inhibit the oncogenic properties of the Myc oncoprotein, which is frequently deregulated in prostate carcinoma. Work from our laboratory has suggested a tumor suppressor role for Bin1, including a role in mediating p53-independent apoptosis by c-Myc in neoplastically transformed cells. A role in prostate cancer was prompted by the finding that the Bin1 gene mapped to the midsection of chromosome 2q, within a region that is deleted in ~40% of metastatic prostate carcinomas. Preliminary data presented in the grant application supported the candidacy of Bin1 as a 2q tumor suppressor gene that is lost or altered during progression to androgen independence, when apoptotic responses are blunted. The specific aims of our proposal were to:

1. Develop monoclonal antibodies that can detect Bin1 in fixed tissues;
2. Perform an immunohistological analysis of Bin1 in staged tumors;
3. Identify mutations in the Bin1 gene in androgen-independent tumor cells; and
4. Investigate the apoptotic potential of Bin1 in androgen-independent cells.

Aims 1 and 2 were completed and significant progress was made on Aims 3 and 4. As a improved approach to examining the effects of Bin1 mutation in prostate cells, we generated a "gene knockout" mouse for Bin1. Preliminary results obtained in this model support the hypothesis that Bin1 loss contributes to the development of hyperplastic abnormalities in the male urogenital tract.

II. Introduction

An important goal in prostate cancer research is to identify better prognostic tools to predict the course and relapse of malignant carcinoma, and to uncover and develop foundations for the development of novel modalities to treat advanced, intractable disease. Malignant prostate adenocarcinomas frequently contain overexpressed c-Myc. Moreover, deregulation is most frequently seen in advanced tumors (due to genetic or epigenetic causes) and, where it occurs, signals poor prognosis. The ability of deregulated Myc to drive apoptosis may provide an Achilles' heel in such cells. Therefore, unraveling Myc death mechanisms represent one direction to address the clinical need for improved therapeutic modalities.

During the course of this project, as part of other work we developed several lines of evidence that support a potential tumor suppressor role for Bin1 in breast cancers and melanoma (Ge et al., 1999; Ge et al., 2000). Mechanistic analyses suggested that the suppressor activities related to the ability of Bin1 to drive apoptosis in tumor cells, and to participate in a mechanism of apoptosis activated by c-Myc in transformed primary cells (DuHadaway et al., 2001; Elliott et al., 2000).

III. Progress on Specific Aims

Aim 1. Develop monoclonal antibodies that detect Bin1 in paraffin-embedded tissues.

Background and Rationale. Initial Bin1 antibodies were specific but not tractable in fixed, paraffin-embedded tissues. Antibodies with this capability are required for retrospective studies, since archival material is preserved in this fashion. They and are also desirable for clinical trials and ultimate diagnostic applications because they are more reliable. Antibodies with higher avidities or that recognize other epitopes were desired to overcome the shortcomings of the initial reagents.

Experimental Approach. Bin1 is a member of the BAR family of adapter proteins, which includes amphiphysin, Bin1, Bin2, and Bin3(the latter three genes were cloned and characterized in our laboratory (Ge and Prendergast, 2000; Routhier et al., 2001; Sakamuro et al., 1996; Wechsler-Reya et al., 1997). Characterization of the Bin1 gene revealed complex patterns of alternate splicing. The so-called BAR domain in Bin1 is found in all splice isoforms. Therefore, we raised monoclonal antibodies by standard methods to the Bin1 BAR domain, which was expressed as a bacterial glutathione-S-transferase (GST) fusion protein. Monoclonal antibodies obtained were typed for specificity by Western and immunoprecipitation analysis. Next, the epitope recognized was mapped using a panel of Bin1 deletion mutants generated in the laboratory. Lastly, antibodies were tested for the ability to stain fixed sections of normal tissues (including prostate tissue) where Bin1 expression had been documented previously by immunohistochemical analysis of frozen tissue sections.

Results. Nine monoclonal antibodies specific for the Bin1 BAR domain were obtained. Two of these antibodies (α-Bin1#6 and α-Bin1#8) were shown to recognize various Bin1 splice isoforms in a panel of fixed and paraffin-embedded tissues. α-Bin1#8 was somewhat more avid using the optimal staining method, which included a steam antigen recovery method that is commonly used in hospital pathology laboratories. Figure 1 (Appendix) shows the staining pattern produced by α-Bin1#8 in normal prostate as well as a panel of prostate tumors (see below). Bin1 is detected primarily in basal epithelial cells, with luminal epithelial cells and stromal cells exhibiting little expression. Low levels of staining in endothelial cells was detected in some sections.

Conclusion. These results confirmed Bin1 expression in prostate epithelial cells and defined two highly specific and avid Bin1 antibodies that are tractable for immunohistochemical analysis of fixed tissue. Both antibodies are being made available to the research community through The Wistar Institute via commercial vendors.

2. Perform an immunohistological analysis of Bin1 in staged primary tumors.

Background. Antibodies tractable for fixed tissue staining were used to assess the frequency of Bin1 loss in prostate cancer, and to determine whether there was any relationship between Bin1 loss and tumor grade. A retrospective study was desirable to determine whether Bin1 status was useful as a prognostic marker.

Experimental approach. During the course of this project we performed two immunohistochemical studies of Bin1 in primary prostate tumors. We also initiated an additional study aimed at assessing the extent of Bin1 loss specifically in prostate tumor metastases. Lastly, we have initiated a study aimed at assessing the possible prognostic utility of Bin1 loss through a retrospective analysis of tumors from patients where there is clinical follow-up information.

The first study was conducted with a panel of 30 cases of frozen primary prostate tumors, in collaboration with Drs. S. Bruce Malkowicz and John T. Tomaszewski at the Hospital of the University of Pennsylvania. This study used the Bin1 antibody 99D, which is tractable only in frozen tissue. This study found that Bin1 was typically expressed in primary tumor cells (29/30 cases). However, analysis of tumor cell lines conducted in parallel studies indicated that both androgen-dependent and androgen-independent cells expressed Bin1, with the latter exhibiting inactivation of Bin1 by a missplicing mechanism. Thus, the staining of primary cells was inconclusive with regard to functional inactivation. In collaboration with Dr. Peter Nelson at the University of Washington, (a member of the CaPCURE tissue consortium), we also examined Bin1 expression by Northern analysis of RNA isolated from 10 cases of prostate tumor metastases. We observed undetectable levels of Bin1 in 10/10 cases, suggesting a selection against Bin1 expression in metastatic disease. These findings were published in 2000 in the *International Journal of Cancer* (Ge et al., 2000).

Using the new antibodies, we have conducted a second study of 50 cases of staged prostate tumors arrayed in a single paraffin block ("tissue array"), in collaboration with Dr. Timothy McDonnell at the M.D. Anderson Cancer Center. The tumors were all primary cancers, variously staged at Gleason grades V (10 tumors), VI (15 tumors), VII (10 tumors), VIII (11 tumors), and IX (4 tumors). We observed marked reduction or undetectable levels of Bin1 in 38/50 (76%) of the tumors in this panel. Interestingly, some positive tumors showed elevation of Bin1 in tumor and stroma suggestive of previous evidence of misspliced Bin1 isoforms in prostate tumor cell lines. Representative immunohistochemical results from this study are presented in Figure 1 (see Appendix). A detailed comparison of Bin1 status to other histopathological markers is currently ongoing. However, preliminary assessments do not identify an obvious link between Bin1 status and tumor grade.

We have initiated an additional study with Dr. McDonnell to look specifically at the rate of Bin1 loss in prostate tumor bone metastases, also using a "tissue array" approach developed in Dr. McDonnell's laboratory to handle bony metastases. Based on previous Northern analysis of RNA from metastases, we predict extensive loss of Bin1 expression.

Recently, we have also extended our collaboration with Drs. Malkowicz and Tomaszewski to perform a retrospective analysis of Bin1 in staged prostate cancers that have clinical follow-up information. Dr. Malkowicz, the urological oncologist in the collaboration, has acquired an extensive panel of tumors with associated follow-up information (~2000). We anticipate that this study will be ongoing throughout 2001.

Conclusion. The results suggest that Bin1 is widely lost in prostate cancers, particular in metastatic disease. To date, Bin1 loss has not been seen to be associated with tumor grade, although further analysis to determine possible association with other pathological markers is ongoing. A recently initiated retrospective analysis may shed light on the potential of Bin1 loss as a prognostic marker.

3. Identify mutations in the Bin1 gene in androgen-independent tumor cells.

Background. Previous work had documented loss-of-heterozygosity (LOH) in 6/15 (40%) of primary prostate tumors examined in collaboration with Drs. Malkowicz and Tomaszewski. The marker used to assess allele number was a microsatellite repeat in intron 5 of the human Bin1 gene. Although the sample size was small, the rate of LOH in this panel was consistent with a previous study that had identified LOH in the mid-2q region of metastatic prostate tumors (Cher et al., 1996). To establish genetic proof for loss of a tumor suppressor, it was necessary to determine whether the remaining allele in the 6 tumors exhibiting LOH was functionally inactivated by mutation.

Experimental approach. We determined the DNA sequence of all the exons in the single allele of Bin1 that remained in the tumors exhibiting LOH.

Results. In 5/6 of these tumors, a consistent alteration (L52Q) was seen in the Bin1 gene in the functionally important BAR domain (in exon 3). However, this alteration was inconclusive, because it was located near the end of the exon where it was consistent with splice site choice alteration or with polymorphism, rather than mutation. In summary, we could not confirm functional elimination of the allele that remained in tumors with LOH. Subsequent immunohistochemical analyses suggest that Bin1 is more likely suppressed at the level of gene shut-off, rather than mutated. Epigenetic suppression is possible given the presence of a canonical CpG island at the Bin1 promoter, although we have not as yet initiated a methylation-specific PCR study that could address this possibility.

4. Investigate the apoptotic potential of Bin1 in androgen-independent cells.

Background. Bin1 messages are expressed in the androgen-dependent LNCaP cell line as well as in the androgen-independent DU145 and PC3 cell lines. However, RT-PCR analysis indicated that, whereas the structure of the LNCaP message was normal, the structure of the DU145 and PC3 messages was abnormal. Specifically, the latter cells expressed a misspliced form of Bin1 that had been documented previously as a loss-of-function event in melanoma cells (Ge et al., 1999). Therefore, it was of interest to examine further the structure of the LNCaP message, and to investigate the effects of re-introducing Bin1 expression into the DU145 and PC3 cell lines. We predicted an apoptotic outcome based on preliminary results in non-prostate tumor cell lines.

Experimental approach. The DNA sequence of the Bin1 messages expressed in each of the cell lines noted above was determined. To assess biological impact, cell lines were transfected by standard methods with Bin1 expression vectors that harbored a neomycin-resistance gene, and the efficiency of G418 colony formation relative to empty vector was compared. In addition, a Bin1 adenoviral vector was used to re-express wild-type Bin1 in DU145 cells. The virus used for this experiment has been described (Elliott et al., 2000). The response to infection by control or Bin1 viruses was compared by flow cytometric analysis and morphological examination.

Results. LNCaP was found to harbor wild-type Bin1. The abnormal splice of the brain-specific exon 12A into Bin1 messages in DU145 and PC3 was confirmed by DNA sequencing. A structural mutation was identified in PC3, but deemed unimportant since 12A missplicing is already sufficient to completely inactivate the antitransforming and proapoptotic activity of Bin1 (Ge et al., 1999). These results were published in 2000 in the *International Journal of Cancer* (Ge et al., 2000)

Colony formation experiments showed that Bin1 overexpression was sufficient to suppress cell outgrowth in LNCaP and PC3 cells, with a several-fold suppression seen in LNCaP and a ~50% suppression in PC3 (Ge et al., 2000). DU145 cells were relatively refractory although an examination of cells from another source suggested susceptibility to inhibition. In support of this likelihood, or possibly reflecting effects of increased transgene expression, infection of DU145 cells with Bin1 adenovirus vector led to manifestations of apoptosis. We did not pursue these results however, because the multiplicity of infection (m.o.i.) that produced this effect was only ~3-fold less than a similar effect produced by control vectors (suggesting that DU145 cells in our experiments were susceptible to nonspecific toxicity of the virus).

5. Grant aims extension: Examination of urogenital status in Bin1 "knockout" mice.

As a more physiological route to investigate the potential role for Bin1 loss in tumor development, we have investigated the status of the urogenital tract in "gene knockout" mice that were generated in the laboratory during the grant period. Homozygous deletion of Bin1 produces embryonic lethality at late times during development in a manner associated with a severe cardiac hyperplasia. At these times no other gross abnormalities have been documented. Although nullizygous animals can not be examined, heterozygous (+/-) mice develop normally and adult mice have been monitored for approximately one year.

Interestingly, starting at ~10 months of age, male +/- mice exhibit progressively increased size. This phenotype is not observed in females. Pathological exams of several male mice that were euthanized have revealed one case of a lymphoma, and several cases of grossly enlarged seminal vesicles. This phenotype appears to reflect gross enlargement and engorgement but also signs of hyperplasic cell growth. The phenotype is unusual but has been reported recently to occur in similarly aged mice that are homozygous for the cell cycle kinase inhibitor p27KIP1 (2001 AACR Meeting). Interestingly, in such mice (which also exhibit increased size) there is also evidence of neoplastic prostate lesions. In support of this observation, Pandolfi's group has recently reported that PTEN +/-; p27KIP -/- mice are strongly prone to prostate cancer (Di Cristofano et al., 2001).

Recent work in our laboratory on Bin1-binding proteins has turned up a biochemical linkage to p27KIP1. This reinforces our interest to pursue the hypothesis with the assistance of a mouse prostate expert, Dr. Cory Abate-Shen (Center for Advanced Biotechnology and Medicine, Rutgers University) that Bin1 +/- male mice may also exhibit lesions in the prostate (which in the case of p27KIP1 -/- mice are not pronounced unless PTEN function is eliminated).

IV. Conclusions

The studies described above indicate that Bin1 expression is frequently lost in prostate cancer, particularly in metastatic disease. This loss may reflect gene deletion,

but appears more frequently to reflect epigenetic mechanisms that include missplicing and gene shut-off. Current work directed at assessing the prognostic significance of Bin1 loss is ongoing. An exciting line of work that has developed is the development of urogenital abnormalities in male mice that have sustained genetic deletion of the Bin1 gene. This line of work has the potential to offer genetic proof of the hypothesis that Bin1 loss plays a causative role in malignancy.

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defects caused by loss of Hob3p, the fission yeast homolog of Rvs161p. J. Biol. Chem. *in press.*

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VI. Publications and Meeting Abstracts

a. Publications citing this grant

Ge, K., DuHadaway, J., Mao, J., Sakamuro, D., Nelson, P., Malkowitz, S.B., Tomaszewski, J.E., and Prendergast, G.C. (2000). Loss of heterozygosity and tumor suppressor activity of Bin1 in prostate adenocarcinoma. Int. J. Cancer **86**, 155-161.

Ge, K. and Prendergast, G.C. (2000). Bin2, a functionally nonredundant member of the BAR adaptor gene family. Genomics **15,** 210-220.

Routhier, E., Abbaszade, I., Summers, M., DuHadaway, J., Burn, T.C., Albright, C., and Prendergast, G.C. (2001). Human Bin3 complements the F-actin localization defects caused by loss of Hob3p, the fission yeast homolog of Rvs161p. J. Biol. Chem., in press.

b. Related publications during the grant period

Elliott, K., Sakamuro, D., Basu, A., Du, W., Wunner, W., Steller, P., Gaubatz, S., Zhang, H., Prochownik, E., Eilers, M., and Prendergast, G.C. (1999). Bin1 functionally interacts with Myc and inhibits cell proliferation through multiple mechanisms. Oncogene **18**, 3564-3573.

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Elliott, K., Ge, K., Du, W., and Prendergast, G.C. (2000). The c-Myc-interacting adaptor protein Bin1 activates a caspase-independent cell death program. Oncogene **19**, 4669-4684.

DuHadaway, J., Sakamuro, D., Ewert. D., and Prendergast, G.C. (2001). Bin1 mediates apoptosis by c-Myc in transformed primary cells. Cancer Res. **61**, 3151-3156.

Routhier, E., Albright, C., and Prendergast, G.C. Hob1+, the fission yeast homolog of the tumor suppressor Bin1, regulates the cell division cycle and the DNA damage response. Manuscript submitted.

c. Abstracts for this project

Abstracts describing the role of Bin1 in prostate cancer were presented at the following symposia and conferences:

2000 Fox Chase Cancer Center, Cancer 2000 Symposium, Philadelphia PA (prostate cancer focus). Invited lecture.
2000 Annual Meeting of Environmental Mutagens Society, New Orleans LA. Invited lecture.
1999 Delaware Valley Prostate Cancer Symposia, Philadelphia PA. Invited lecture.
1999 15th Annual Meeting on Oncogenes, Hood College, Frederick MD. Poster.

d. Related abstracts

Abstracts describing other Bin1-related research were presented at the following conferences:

2001 2nd International Conference on Mechanisms of Cell Death and Disease: Advances in Therapeutic Invention, North Falmouth MA. Invited lecture (scheduled).
2000 AACR Annual Meeting (*Apoptosis: Mechanisms*), San Francisco CA. Lecture.
1999 AACR Annual Meeting (*Apoptosis*), Philadelphia PA. Lecture.
1999 Cold Spring Harbor Meeting on Programmed Cell Death, Cold Spring Harbor NY. Lecture.
1999 15th Annual Meeting on Oncogenes, Hood College, Frederick MD. Other posters and lecture.

e. Patents

U.S. Patent Application 60/151,554. "Bridging Integrator-2 (Bin2) nucleic acid molecules and proteins and uses therefor". Claims: Bin2 nucleic acid and amino acid sequences. Pending.

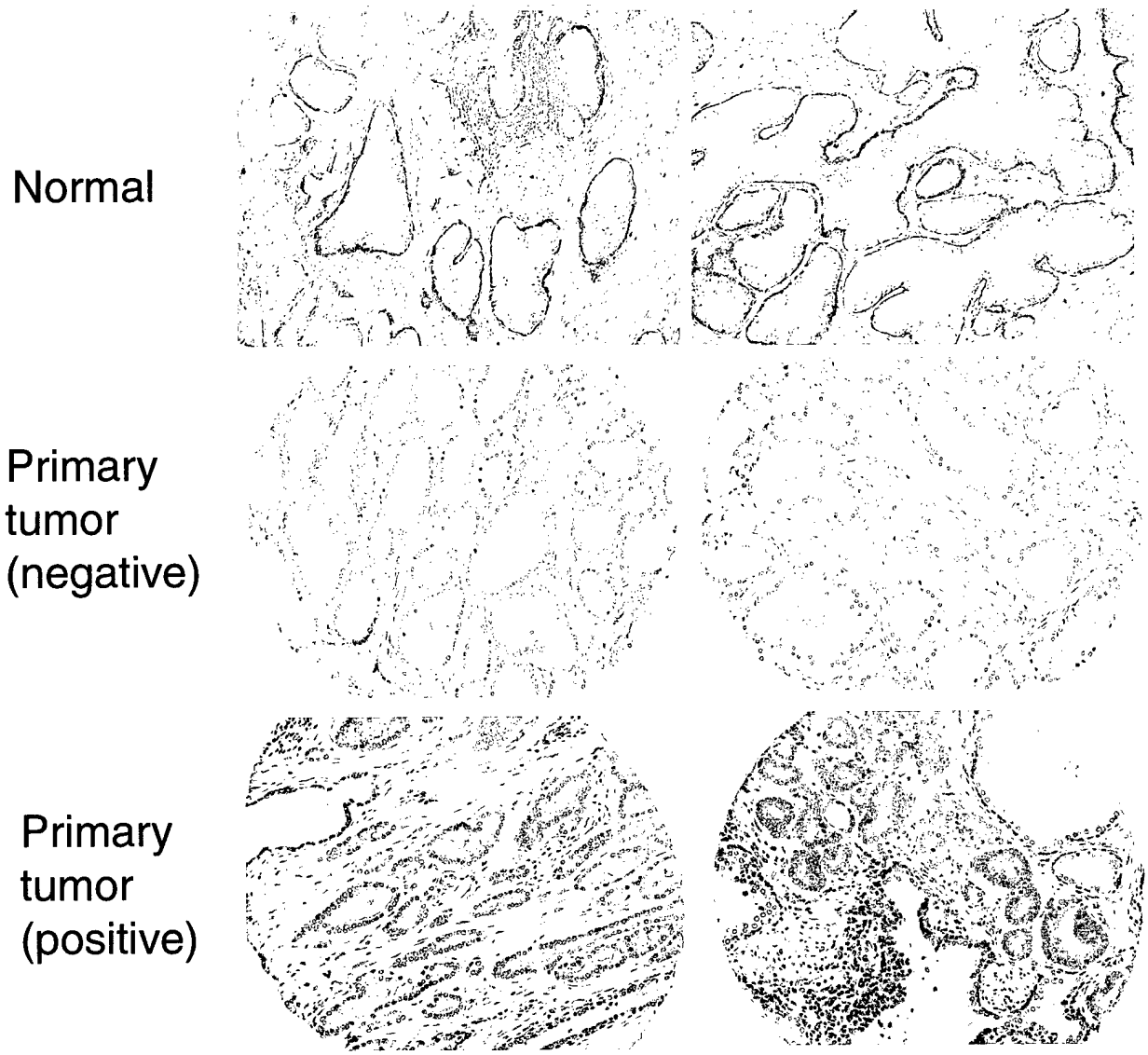
VII. Appendices

Figure 1. Immunohistochemical analysis of Bin1 in benign and tumorous prostate tissues. Fixed tissue arrayed in a paraffin block was processed with monoclonal antibody α-Bin1#8 as the primary antibody. Representative samples are shown.

Reprints (2)

Preprints (1)

FIGURE 1



Int. J. Cancer: **86,** 155–161 (2000)

Publication of the International Union Against Cancer

LOSS OF HETEROZYGOSITY AND TUMOR SUPPRESSOR ACTIVITY OF *BIN1* IN PROSTATE CARCINOMA

Kai GE[1], Farooq MINHAS[2], James DUHADAWAY[1,3], Nien-Chen MAO[1], Darren WILSON[2], Roberto BUCCAFUSCA[1], Daitoku SAKAMURO[1], Peter NELSON[4], S. Bruce MALKOWICZ[5], John TOMASZEWSKI[2] and George C. PRENDERGAST[1,3]*

[1]*The Wistar Institute, Philadelphia, Pennsylvania, USA*
[2]*Department of Pathology and Laboratory Medicine, Hospital of the University of Pennsylvania, Philadelphia, PA, USA*
[3]*DuPont Pharmaceuticals, Glenolden Laboratory, Glenolden, PA, USA*
[4]*Department of Molecular Biotechnology, University of Washington, Seattle, WA, USA*
[5]*Department of Urology, Hospital of the University of Pennsylvania, Philadelphia, PA, USA*

The genetic events underlying the development of prostate cancer are poorly defined. c-Myc is often activated in tumors that have progressed to metastatic status, so events that promote this process may be important. Bin1 is a nucleocytoplasmic adaptor protein with features of a tumor suppressor that was identified through its ability to interact with and inhibit malignant transformation by c-Myc. We investigated a role for Bin1 loss or inactivation in prostate cancer because the human *Bin1* gene is located at chromosome 2q14 within a region that is frequently deleted in metastatic prostate cancer but where no tumor suppressor candidate has been located. A novel polymorphic microsatellite marker located within intron 5 of the human *Bin1* gene was used to demonstrate loss of heterozygosity and coding alteration in 40% of informative cases of prostate neoplasia examined. RNA and immunohistochemical analyses indicated that *Bin1* was expressed in most primary tumors, even at slightly elevated levels relative to benign tissues, but that it was frequently missing or inactivated by aberrant splicing in metastatic tumors and androgen-independent tumor cell lines. Ectopic expression of *Bin1* suppressed the growth of prostate cancer lines *in vitro*. Our findings support the candidacy of *Bin1* as the chromosome 2q prostate tumor suppressor gene.
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The genetic causes of prostate cancer remain largely unknown. While common in men, prostate tumors are generally both localized and indolent, so genetic events that predispose or promote conversion to an aggressive, metastatic status are of particular interest. One of the most common chromosomal abnormalities seen in tumors that have acquired invasive and metastatic potential is gain of chromosome 8p, where the c-*Myc* gene is located (Bova and Isaacs, 1996). Gains of 8q are well-correlated with disease progression insofar as they are found in 85% of lymph node metastases and in 89% of recurrent hormone refractive tumors (Cher *et al.,* 1996; van den Berg *et al.,* 1995; Visakorpi *et al.,* 1995). c-*Myc* amplification or overexpression is found in many prostate tumors and is a likely progression marker (Buttyan *et al.,* 1987; Fleming *et al.,* 1986; Jenkins *et al.,* 1997). Oncogenic activation of c-Myc by gene amplification delivers a powerful signal that is sufficient to drive cell cycle progression and malignant growth in many types of cells, including prostate cells (Thompson *et al.,* 1989). However, in premalignant cells, c-Myc can also activate apoptosis such that its oncogenic activation is balanced by apoptotic penalty (Prendergast, 1999). Therefore, malignant cells may escape this penalty by inactivating tumor suppressor functions. p53 is an important player but is likely irrelevant to this process in prostate cells because inactivation of p53 does not compromise c-Myc-mediated apoptosis in epithelial cells (Sakamuro *et al.,* 1995; Trudel *et al.,* 1997). Thus, loss or inactivation of molecules other than p53 should be considered.

c-Myc lies at an intersection of 2 signaling networks that target its C-terminal DNA binding domain and its N-terminal transcriptional transactivation domain (Sakamuro and Prendergast, 1999). The major player in the C-terminal network is Max, a helix-loop-helix/"leucine zipper" (HLH/Z) protein that heterodimerizes with c-Myc and mediates its ability to specifically recognize DNA. However, Max alterations do not occur in any human cancer, including prostate cancer. The Max-binding protein Mxi1, which can titer Max away from c-Myc, has been reported to be altered in prostate tumors, but this event appears to represent at best only a fraction of tumors where c-Myc is overexpressed. Recent advances have led to the identification of a set of novel N-terminal-interacting proteins that constitute a second Myc network (Sakamuro and Prendergast, 1999). One of these proteins, Bin1 (Bridging INtegrator 1), is a ubiquitous adaptor protein that has features of a tumor suppressor (Elliott *et al.,* 1999; Sakamuro *et al.,* 1996) and that has been linked to cell death and differentiation decisions (Prendergast, 1999; Sakamuro and Prendergast, 1999). We hypothesized that Bin1 might be inactivated in prostate cancer because the human *Bin1* gene maps to chromosome 2q14 (Negorev *et al.,* 1996), within a region of chromosome 2q that is frequently deleted in metastatic prostate tumors (Cher *et al.,* 1996) but where no tumor suppressor gene has been identified to date. Since c-*Myc* is frequently amplified in prostate carcinomas and Bin1 can suppress malignant transformation by c-Myc (Sakamuro *et al.,* 1996), loss of Bin1 activity would eliminate a mechanism that can limit full oncogenic activation of c-Myc. Our results suggest that loss or inactivation of the *Bin1* gene occurs frequently in prostate cancer, in support of its candidacy for the 2q prostate tumor suppressor gene.

MATERIAL AND METHODS

LOH analysis

Normal lymphocyte genomic DNAs were a gift from H. Reithman (Philadelphia, PA). Genomic DNAs were isolated by standard methods from microdissected malignant and adjacent normal tissues obtained from the Department of Urology at the University of Pennsylvania (Philadelphia, PA). For PCR analysis, an approximately 141 bp fragment containing a $(TG)_{17}$ microsatellite identified in intron 5 was amplified by 35 cycles of PCR. Oligonucleotide primers were 5′-TTTCTGAGGCAGGCTTCCCACTTC and 5′-CGTCTGTGTGAAGAGGTGTGTG. Reactions were per-

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Farooq Minhas's present address is: Shadyside Hospital, University of Pittsburgh Medical Center, Department of Pathology, Pittsburgh, Pennsylvania, USA.

Daitoku Sakamuro's present address is: Department of Medicinal Chemistry, School of Pharmacy and Pharmaceutical Sciences, Purdue University, West Lafayette, Indiana, USA.

*Correspondence to: Glenolden Laboratory, 500 South Ridgeway Avenue, Glenolden, PA, USA 19036. Fax: +1 610 237 7837.
E-mail: george.c.prendergast@dupontpharma.com

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formed in 50 μl and contained 60 mM TrisCl pH 10/1.5 mM $MgCl_2$/15 mM NH_4SO_4/1 mM dNTPs/1U Taq polymerase including 1 μl 800 mCi/mmol [^{32}P]-dCTP. The PCR cycle was 1 min at 94°C/30 sec at 54°C/30 sec at 72°C. Reaction products (4 μl) were examined on agarose gels to verify the expected product and then fractionated on a standard 6% sequencing gel, dried and autoradiographed.

Northern analysis

Prostatic tissues were obtained through the CaPCURE Tissue Consortium at the University of Washington (Seattle, WA). Total cytoplasmic and total cellular RNAs were isolated from cell lines and tissues and analyzed by Northern blotting. Briefly, 10 μg RNA per lane was fractionated on 1.0% agarose gels, blotted to Duralon membranes (Stratagene, La Jolla CA) and hybridized to human *Bin1* or c-*Myc* cDNA probes.

RT-PCR

One microgram total cytoplasmic RNA in 5 μl water was denatured by a 5-min incubation at room temperature with 2 μl 0.1 M methylmercury hydroxide. To this RNA were added 2.5 μl 0.7 M β-mercaptoethanol and 0.5 μg random hexanucleotides in 1 μl aqueous solution. Following a 2-min incubation at 70°C, RNAs were incubated on wet ice, and 2.0 ml 5 μM dNTPs, 0.5 μl RNase inhibitor (Promega, Madison WI), 4 μl 5× RT buffer, 2 μl 0.1 M DTT and 1.0 μl Mo-MLV reverse transcriptase (Life Technologies, Gaithersburg, MD) were added. Reactions were incubated 60 min at 37°C and then stopped by a 5-min incubation at 95°C. This RT product was used as template to amplify *Bin1* or β-actin cDNA by PCR. For *Bin1* (exons 1–5), the 5′ primer was 5′-AAAGATCGCCAGCAACGTGC and the 3′ primer was 5′-CTGGTGGTAATCCATCCACAGC. For β-actin (exons 3–4), the 5′ primer was 5′-GGTGGGCATGGGTCAGAAGG and the 3′ primer was 5′-GCAGCTCGTAGCTCTTCTCC. Reactions were performed in 100 μl containing 200 ng template, 50 pmol each primer, 0.4 mM each dNTP, 1× Taq PCR buffer (BMB) and 2.5 units Taq polymerase. PCR was performed by a 2-min incubation at 96°C and then 26 cycles (*Bin1*) or 22 cycles (β-actin) of 30 sec at 96°C/45 sec at 61°C/45 sec at 72°C followed by a final extension of 10 min at 72°C. Products were purified from 1.4% TBE agarose gels and in some cases subjected to direct DNA sequencing. For RNA samples in which the entire coding region of *Bin1* cDNA was cloned by RT-PCR and subjected to direct DNA sequencing, 3 separate PCR reactions were performed using primers and conditions that have been described (Wechsler-Reya *et al.,* 1997*b*).

Immunohistochemistry

Tissue obtained from 30 radical prostetectomy specimens of patients undergoing surgery at the Hospital of the University of Pennsylvania was used. The radical prostectomy specimens were inked and serially sectioned, and areas grossly suspicious for prostatic adenocarcinoma and adjacent benign tissue were taken. Frozen sections were immediately obtained or the tissues were stored at −70°C after snap-freezing in liquid nitrogen. The tumors were classified using the Gleason grading system. In addition, in each section, prostatic intraepithelial neoplasia (PIN) was identified, and benign prostatic tissue was subcategorized where appropriate as benign prostatic hyperplasia (BPH) or atrophy. Frozen tissue sections were warmed to room temperature, washed twice with PBS, incubated in 4% paraformaldehyde/PBS for 30 min at 4°C, rinsed in water and then twice more with PBS. Tissues were permeabilized in 0.1% Triton-X-100 for 10 min, washed twice again with PBS and incubated for 15 min in 1% H_2O_2 in methanol to quench endogenous peroxidase. After washing in PBS and then in PBS/0.1% BSA for 5 min, samples were incubated for 30 min with anti-Bin1 monoclonal antibody (MAb) 99D (Wechsler-Reya *et al.,* 1997*a*), washed twice in PBS and incubated an additional 30 min with the secondary antibody diluted at 1:200 in PBS/0.1% BSA without sodium azide (Biotin-SP-conjugated AffiniPure Goat Anti-Mouse IgG(H+L), Jackson ImmunoResearch, Rockland, PA, 115-065-062). To develop the slides, samples were incubated 30 min with tertiary reagent diluted at 1:200 in PBS/0.1% BSA without azide (peroxidase-conjugated streptavidin, DAKO, Carpinteria, CA, P-0397), washed in PBS 3 times for 5 min, then the slides were flooded 5 min with substrate (Peroxidase Substrate Kit DABkit, Vector, Burlingame, CA, SK-4100). After rinsing in water, slides were counterstained with 0.04% light green in acidified water or dilute hematoxylin for approximately 1 min, dehydrated and cover-slipped. 99D efficiently stained sections from frozen tissue but not tissues that were fixed before sectioning.

Cell culture

LNCaP, PC3 and DU145 cells were obtained from ATCC. Cells were cultured in DMEM containing 10% FCS (Life Technologies) and penicillin/streptomycin (Fisher, Pittsburgh, PA). Cells were transfected using a standard calcium phosphate precipitation method. Briefly, 2×10^5 cells seeded in 10-cm dishes were transfected overnight (18 hr) with 20 μg empty pcDNA3.1 vector (Invitrogen, La Jolla, CA) or the human *Bin1* vector CMV3-Bin1 (Elliott *et al.,* 1999). The next day, cells were washed and refed with growth media. After an additional 24 hr, cells were trypsinized and passaged at 1:3 or 1:6 or into new dishes, and the following day G418 was added to 0.5 mg/mL for selection of stable transfectants. Colonies were scored by methanol fixation and crystal violet or Coomassie blue staining 2–3 weeks later.

RESULTS

Loss-of-heterozygosity (LOH) analysis was performed with genomic DNAs isolated from a set of malignant and patient-matched normal prostate tissues as follows. The marker was a novel dinucleotide microsatellite $(TG)_{17}$ sequence that was identified in intron 5 of the human *Bin1* gene during its characterization (Wechsler-Reya *et al.,* 1997*b*). Examination of this sequence in genomic DNAs isolated from the peripheral blood lymphocytes of 12 unrelated individuals indicated the existence of several alleles. The informative content of the marker indicated utility for LOH analysis insofar as heterozygosity was observed in 10/12 (83%) of the samples examined (Fig. 1*a*). Genomic DNAs isolated from

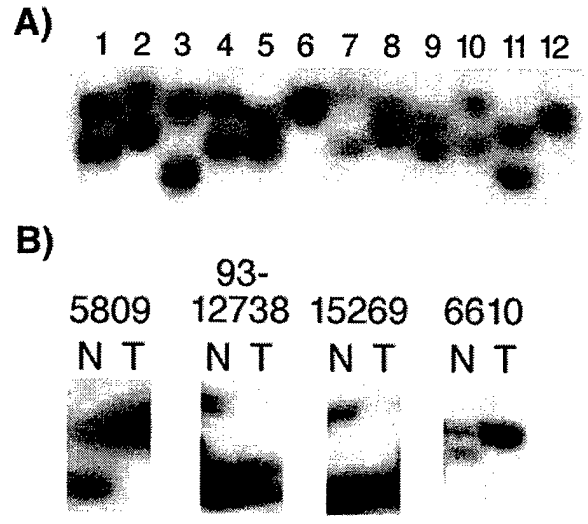

FIGURE 1 – Allelic deletion of Bin1 in prostate cancer. (*a*) Definition of a polymorphic microsatellite within intron 5 of the human *Bin1* gene. PCR products were checked on agarose gels to verify the expected size and abundance and then fractionated on a 6% sequencing gel and autoradiographed. (*b*) Examples of LOH in prostate tumor DNAs. Genomic DNAs isolated from microdissected malignant tissues and matched normal prostate tissues were subjected to PCR and gel fractionation as above. Representative results are shown from 4 informative pairs exhibiting LOH.

cells microdissected from 19 mid-range Gleason score tumor and adjacent normal tissues were analyzed for heterozygosity of the marker (Table I, Fig. 1*b*). In this panel, 15/19 pairs were informative for the marker, and 6/15 (40%) of the informative pairs exhibited LOH. The allelic losses observed were specific because a similar analysis of 18 informative bladder cancer DNAs showed no cases of LOH (Table I). The frequency of LOH in prostate cancer was in excellent agreement with the results of Cher *et al.* (1996), who identified deletions at mid-2q with a frequency of 42%. The DNA sequence of the 15 non-neuronal exons of the remaining allele was determined in the 6 samples that exhibited LOH. A consistent and nonconservative coding region alteration was observed in 5/6 of these samples at a residue that was evolutionarily conserved (L52Q) and located in the functionally important N-terminal BAR region (Elliott *et al.,* 1999). The similarity of this variation was not due to cross-contamination of the samples because other alterations were present that did not affect coding potential (*i.e.*, at wobble bases in the codon) but which were unique to each individual sample. Analysis of genomic DNAs from other malignant prostate tissues revealed cases of similar alterations (data not shown), suggesting the possibility of polymorphism. In any case, the frequency and occurrence of this alteration within a functionally crucial region of *Bin1* supported the possibility that malignant phenotypes may be affected at some level.

We next examined the status of *Bin1* expression by performing Northern, RT-PCR and immunohistochemical analyses of a panel of benign, malignant and metastatic prostate tissues and of the prostate cancer cell lines LNCaP, PC3 and DU145. Northern analysis of benign prostate tissues showed that RNA levels were similar to those seen in placenta (a positive control tissue), consistent with previous observations that suggest ubiquitous expression of this gene (Sakamuro *et al.,* 1996). Primary tumors exhibited relatively similar or modestly reduced levels of Bin1 message when normalized to levels of 18S ribosomal RNA (Fig. 2*a*). An exception was seen in sample 2017R, where Bin1 message was undetectable. In contrast, both metastatic lesions examined in this panel lacked detectable Bin1 RNA (Fig. 2*a*). This observation was confirmed and extended by analysis of a second set of metastatic tumors, which suggested that losses of *Bin1* at this stage of progression occurred frequently (Fig. 2*b*). Hybridization of the same Northern blot with a c-*Myc* probe showed that *Bin1* losses occurred in 4/7 metastatic lesions that overexpressed c-*Myc* but also in the remaining lesions that lacked this event (Fig. 2*b*). The extent of such losses therefore extended broadly, a finding consistent with evidence from structure-function studies indicating that Bin1 has both Myc-independent and Myc-dependent tumor suppressor properties (Elliott *et al.,* 1999).

To examine Bin1 protein levels in prostate cancer, we performed an immunohistochemical analysis of 30 cases of mid-Gleason-grade primary prostate tumors that included tumorous and benign tissues (Fig. 3, Table II). This analysis was conducted with anti-Bin1 MAb 99D, which stains frozen but not fixed tissues, limiting the analysis to primary tumors. Stroma and atrophic cells characteristic of aging tissues stained weakly relative to epithelial cells in benign prostatic hyperplasia (BPH), which were uniformly positive (Fig. 3*ad*) in the same fashion as epithelial cells in normal tissues (data not shown). The nuclear staining pattern observed was consistent with previous studies of Bin1 localization in tissues and tissue culture cells. Prostatic adenocarcinoma and prostatic intraepithelial neoplasia (PIN), a preneoplastic lesion, also exhibited positive nuclear staining (Fig. 3*e–h*). Nuclear counts indicated that cancerous cells stained slightly more strongly than BPH cells (Table I). Statistical analysis of these data established that the difference between cancerous and BPH and especially atrophic cells was statistically significant (Fig. 4). The trend in the immunohistochemical results, while modest, differed from that seen by Northern analysis, possibly reflecting the effects of tumor heterogeneity (which is quite significant in prostate malignancy) or differences in protein stability. Nevertheless, the immunochemical results clearly indicated that *Bin1* is expressed in benign as well as

TABLE I – LOSS OF HETEROZYGOSITY ANALYSIS

Tissue type	Informative samples	Allelic losses	Percent LOH
Prostate	15	6	40
Bladder	18	0	0

Genomic DNAs isolated from a set of tumorous and patient-matched benign tissues were analyzed for heterozygosity of a dinucleotide microsatellite $(TG)_{17}$ sequence located in intron 5 of the human *Bin*1 gene (Wechsler-Reya *et al.,* 1997*b*). Prostate tumors were mid-range Gleason-grade primary adenocarcinomas. DNAs isolated from bladder tissues were used as a control to address the specificity of allelic losses.

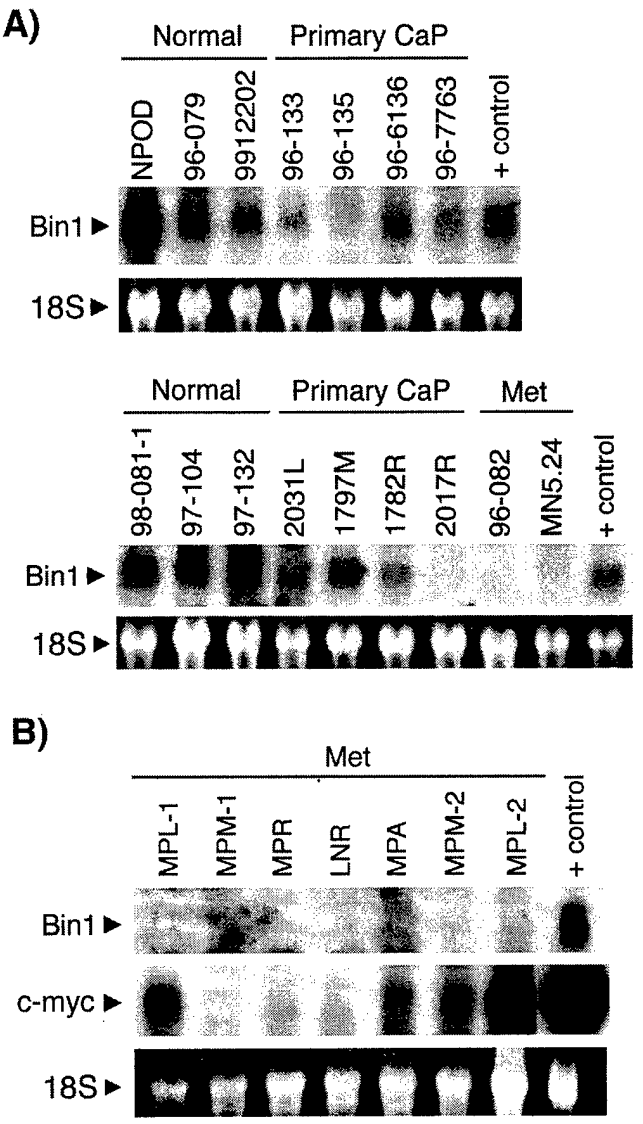

FIGURE 2 – Frequent losses of Bin1 message in metastatic prostate tissues. (*a*) Bin1 expression in a panel of normal, primary tumor and metastatic human prostate tissues. Total RNAs isolated from tissue samples indicated were subjected to Northern analysis using a *Bin1* cDNA probe (Sakamuro *et al.,* 1996). Placental RNA was used as a positive control for detection of Bin1 RNA. Met: metastatic tumors. (*b*) Losses in metastatic tumors are frequent and extend to tumors where c-Myc is not overexpressed. The Northern blot was sequentially hybridized with *Bin1* and human c-*Myc* cDNA probes to identify tumors where c-Myc was overexpressed.

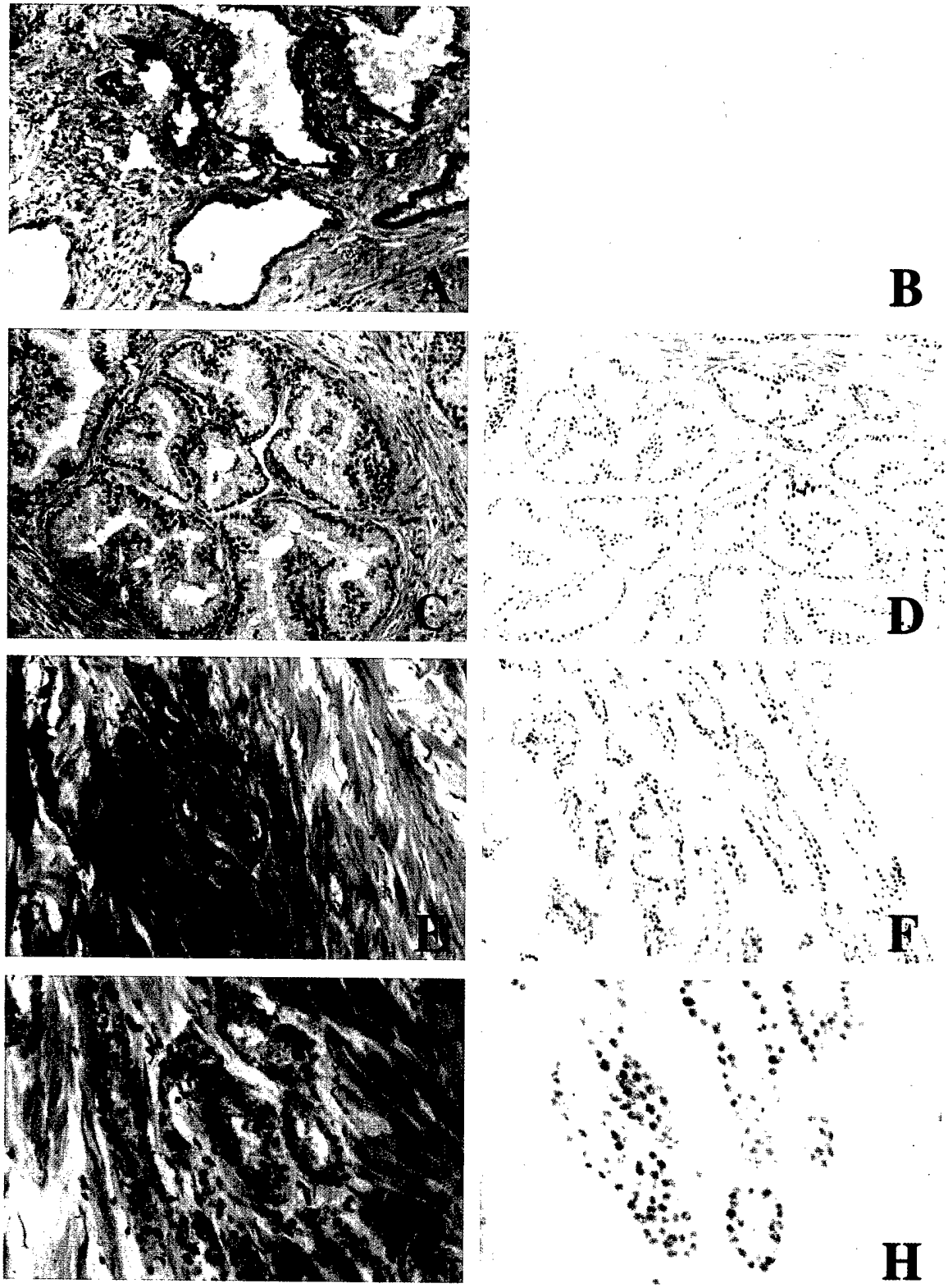

FIGURE 3 – Immunohistochemical analysis. Thirty frozen cases of primary prostate adenocarcinoma that included normal tissue, benign prostatic hyperplasia (BPH), prostate intraepithelial neoplasia (PIN) and prostatic atrophy were subjected to immunohistochemistry with the anti-Bin1 MAb 99D (Wechsler-Reya *et al.*, 1997*a*). Adjacent sections were stained with H&E (*a,c,e,g*) or processed for 99D staining (*b,d,f,h*). Stromal cells stained lightly for the Bin1 antigen(s) under the conditions employed. The quantitative results of staining intensities observed in each tissue are presented in Table II and Figure 4. (*a,b*) Atrophy. Little to no staining is evident. (*c,d*) Benign hyperplasia. Epithelial cell nuclei are stained to a degree similar to that seen in a variety of normal tissues including prostate. (*e,f,g,h*) Prostatic adenocarcinoma. Nuclear staining is retained in both PIN and primary malignant cells.

TABLE II – IMMUNOHISTOCHEMICAL ANALYSIS[1]

Case number	Gleason grade	CaP		BPH		PIN		Atrophy	
		Bin1 score	Number of positive nuclei	Bin1 score	Number of positive nuclei	Bin1 score	Number of positive nuclei	Bin1 score	Number of positive nuclei
1	5+4	3+	130/500	2+	70/500				
2	3+4	4+	410/500						
3	3+3	4+	460/500						
4	3+4	3+	455/500	2+	205/500	3+	420/500	1+	60/500
5	4+4	4+	380/500	2+	245/500			1+	10/500
6	3+3	4+	405/500	1+	190/500				
7	3+3	4+	435/500	3+	365/500				
8	3+4	4+	480/500	2+	260/500			1+	70/500
9	3+4	4+	418/500	3+	360/500				
10	3+3	4+	398/500	3+	265/500				
11	4+3	4+	432/500	3+	370/500				
12	2+3	4+	322/500	3+	290/500			1+	70/500
13	3+3	4+	446/500	1+	50/500			1+	30/500
14	3+3	4+	480/500	3+	390/500				
15	4+5	3+	385/500	3+	310/500				
16	3+3	3+	385/500	3+	345/500			1+	85/500
17	3+3	3+	390/500	2+	290/500			1+	80/500
18	3+3	4+	410/500	3+	355/500	3+	385/500		
19	3+4	4+	392/500	1+	155/500	4+	343/500	1+	25/500
20	3+3	3+	380/500	1+	60/500				
21	3+3	4+	320/500	3+	210/500			1+	80/500
22	3+4	3+	415/500	3+	317/500			1+	35/500
23	3+4	0+	05/500	1+	50/500				
24	3+4	4+	430/500	2+	170/500			1+	25/500
25	3+3	4+	351/500	2+	170/500	3+	305/500	1+	23/500
26	3+4	4+	358/500	2+	160/500	3+	240/500	1+	30/500
27	3+3	4+	442/500	2+	195/500			1+	18/500
28	3+3	3+	365/500	3+	320/500			1+	35/500
29	3+3	4+	357/500	3+	300/500	4+	350/500	1+	60/500
30	3+3	4+	354/500	2+	210/500	3+	295/500	1+	40/500

Frozen tissues obtained from 30 radical prostetectomy specimens of patients undergoing surgery for prostatic adenocarcinoma were examined. Tissue sections examined included both benign and tumor tissue. Slides were scored by 2 observers based on the mean extent of nuclear Bin1 staining, with 500 cells of each tissue type counted. Zero (0) was assigned for no positivity of cells in the section; 1 for <2% positivity; 2 for 2%–10% positivity; 3 for 10%–50% positivity and 4 for >50% positivity of tumor, PIN or BPH as appropriate. For statistical analysis, only those cases with a score of 3 or above were considered positive, with the remainder classified as negative or weak. The ANOVA algorithm in Stat View V1.03 software was used for statistical analysis of the results. CaP: cancer of the prostate; BPH: benign prostatic hyperplasia; PIN: prostatic intraepithelial neoplasia; atrophy: atrophic or aging cells sometimes mistaken for malignant cells in tissue biopsy sections.

[1]For $p < 0.05$: CaP *vs.* BPH: significant; CaP *vs.* PIN: not significant; CaP *vs.* atrophy: significant; BPH *vs.* PIN: not significant; BPH *vs.* atrophy: significant; PIN *vs.* atrophy: significant.

primary tumor cells at the protein level, suggesting that losses and/or inactivation may occur mainly in metastatic tumors.

Additional support for this conclusion was provided by an examination of Bin1 structure and expression in the human prostate cancer cell lines LNCaP, DU145 and PC3. LNCaP cells expressed the highest steady-state levels of message, but Bin1 RNA was also detectable in PC3 and DU145 (Fig. 5, lower panels). RT-PCR and DNA sequencing was performed to examine coding region structure and splice patterns because the *Bin1* gene is subjected to tissue-specific splicing (Wechsler-Reya *et al.*, 1997*b*). This analysis showed that androgen-dependent LNCaP cells express the c-Myc-binding Bin1 isoform, which includes exon 13. This exon is constitutively alternately spliced in all normal cells and encodes approximately half of the c-Myc-binding domain. In contrast to LNCaP cells, the androgen-independent PC3 and DU145 cells poorly expressed exon 13 and expressed instead the brain-specific exon 12A (Fig. 5, upper panels). Mis-splicing of exon 12A in the absence of the other brain-specific exons 12B–12D, as seen in PC3 and DU145, has been shown in melanoma to be a nonphysiological and tumor-specific event that abolishes tumor suppressor activity and eliminates the ability of Bin1 to inhibit malignant transformation by c-*Myc* or adenovirus E1A (Ge *et al.*, 1999). Thus, whereas LNCaP cells retain a c-Myc-interacting isoform of Bin1, PC3 and DU145 cells express a Bin1 isoform(s), which lacks the capacity to bind and/or suppress the oncogenic activity of c-Myc. Since acquisition of androgen independence is a characteristic feature of prostate tumors with metastatic capability, the expression of exon 12A isoform(s) in PC3 and

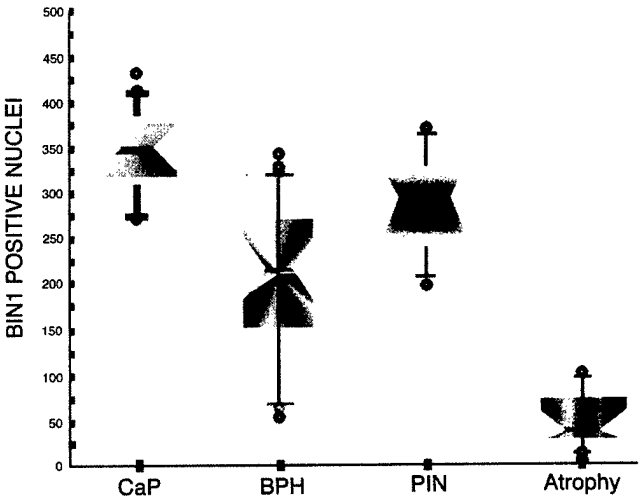

FIGURE 4 – Quantitation of Bin1 levels determined by immunohistochemistry. The distribution and standard error of data shown in Table II were computed and plotted to compare the relative level of Bin1 staining in cells of each pathological type examined. Atrophic cells show a statistically significant suppression in Bin1 levels relative to the other cell types. The < 2-fold increase in Bin1 levels observed in cancerous (CaP) cells relative to BPH cells was also statistically significant in this analysis.

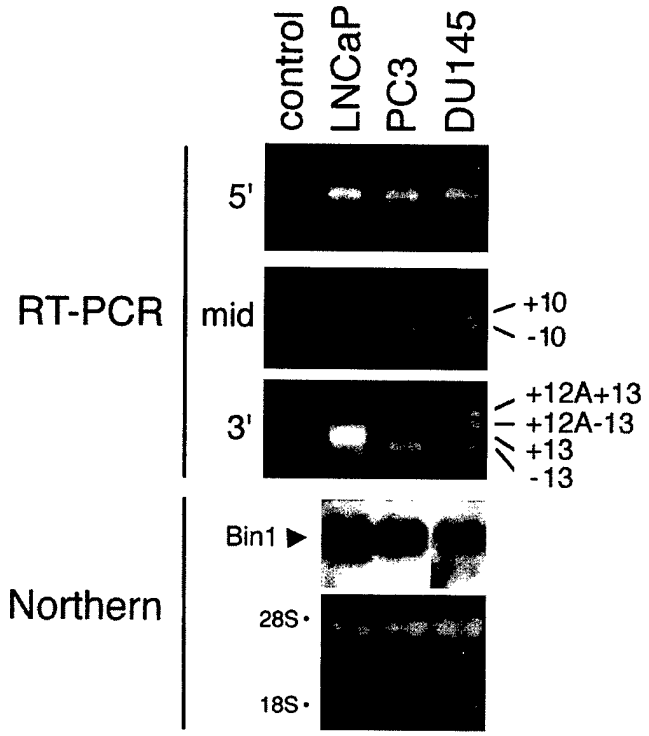

FIGURE 5 – Aberrant splicing of Bin1 in androgen-independent prostate cell lines. Total cytoplasmic RNA isolated from androgen-dependent LNCaP cells and androgen-independent PC3 and DU145 cells were examined by Northern and RT-PCR analyses as described in Material and Methods. The DNA sequence of the complete coding region of each cDNA was determined. Splice pattern and amino acid coding sequences of *Bin1* were wild type in LNCaP. Whereas DU145 had silent alterations but no coding mutations, PC3 included a conservative coding alteration (L214V) within the central U1 region (Elliott *et al.,* 1999). Bottom panels: Northern analysis showing detectable reduction of *Bin1* message in PC3 and DU145 relative to LNCaP cells. Top panels: Results of RT-PCR analysis across 3 separate segments of the cDNA encompassing the complete coding region. Differences in splice isoforms are apparent in the middle (mid) and 3′ regions of *Bin1* cDNA, involving the muscle-specific exon 10 and the brain-specific exon 12A (Wechsler-Reya *et al.,* 1997*b*). Exon 13 encodes part of the c-Myc-binding domain of Bin1. Although constitutively alternately spliced in all normal cells and LNCaP, PC3 and DU145 cells express lower or undetectable levels of the exon 13-containing (13+) isoforms that are capable of c-Myc interaction. Exon 10 splicing in DU145 is an aberrant event but not one that compromises the tumor suppressor activity of Bin1 (Elliott *et al.,* 1999). Splicing of exon 12A in the absence of the brain-specific exons 12B, C and D, which is observed in PC3 and DU145 but not in LNCaP cells, has been shown to be a nonphysiological and tumor-specific event that abolishes the tumor suppressor activity of Bin1 (Ge *et al.,* 1999). Control: RT-PCR performed in the absence of added RNA.

DU145 supports the hypothesis that Bin1 is targeted for loss or inactivation at advanced progression stages, because LNCaP is androgen-dependent whereas PC3 and DU145 are androgen-independent. Ectopic expression of the c-Myc-interacting Bin1 isoform caused a reduction in the relative efficiency of G418-resistant colony formation in PC3 and LNCaP cells but not in DU145 cells (Fig. 6). While the reason for the lack of effect in the latter cell line was unclear, DU145 are resistant to many antiproliferative stimuli, and their resistance provided evidence that Bin1 was not toxic *per se* to prostate cells. In summary, the results supported the conclusion that Bin1 had a negative role in prostate cell growth and was inactivated at some level in prostate cells and that loss or inactivation was likely to have biological significance.

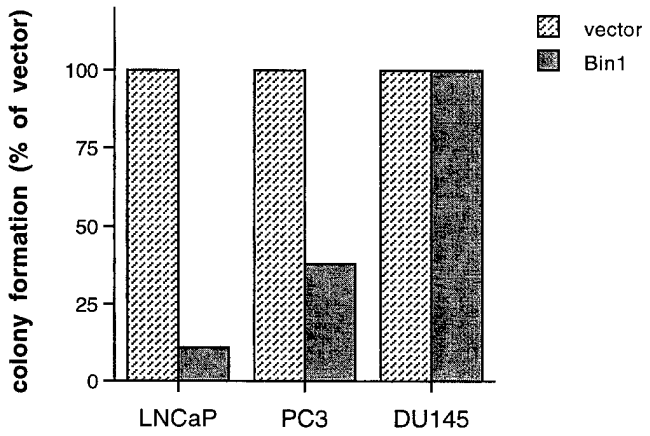

FIGURE 6 – Tumor suppressor activity of Bin1 in prostate cells. Cells were transfected with 20 μg of the neomycin-resistance gene-marked vectors CMV3-Bin1 or pcDNA3 (empty vector), and stable transfectants were selected by culturing in growth media containing G418. Drug-resistant colonies were scored approximately 3 weeks later by fixation in methanol and staining with crystal violet. The colony formation data are presented as the proportion of colonies formed by CMV3-Bin1 relative to those formed by empty vector alone. LNCaP and PC3 exhibit a growth suppression revealed by a reduction in colony formation following ectopic Bin1 expression, whereas DU145 is not affected.

DISCUSSION

Our report identifies the *Bin1* gene as a candidate for the prostate tumor suppressor gene on the middle of the long arm of chromosome 2 (Cher *et al.,* 1996). Allelic deletion and a common alteration was documented at the *Bin1* locus in 40% of a panel of primary prostate tumors examined. We found that LOH in primary tumors was not associated with losses in RNA or protein levels but rather with persistent expression at levels even slightly higher than that seen in benign cells. The basis for the latter was unclear. Alterations of *Bin1* such as the L52Q alteration seen in samples exhibiting LOH might be germane, but their extent and biological consequences, if any, remain to be established. In support of the notion that allelic deletions occur in primary tumors, which are usually androgen-dependent, the androgen-dependent cell line LNCaP has been reported to lack one copy of chromosome 2 (Hyytinen *et al.,* 1997). In contrast to primary tumors, metastatic lesions and androgen-independent cell lines exhibited loss or inactivation of *Bin1*. Losses in metastatic lesions suggest a role for *Bin1* at a later stage in progression when cells acquire the ability to invade and survive in the absence of appropriate adhesion and hormonal signals. The frequency of losses seen extended beyond tumors exhibiting c-Myc overexpression, a feature that may reflect the fact that Bin1 also has c-Myc-independent tumor suppressor activities (Elliott *et al.,* 1999). The inactivation of *Bin1* seen in androgen-independent cell lines occurred by an aberrant splicing event identical to that shown to cause loss of Bin1 tumor suppressor activity in melanoma (Ge *et al.,* 1999). This splice event introduces brain-specific sequences into Bin1, abolishing its ability to suppress malignant transformation by c-Myc or by adenovirus E1A, which acts independently of c-Myc (Ge *et al.,* 1999). Thus, both the c-Myc-dependent and c-Myc-independent tumor suppressor activities of Bin1 are eliminated by aberrant splicing.

The function of Bin1 is complex and involves splice isoform-specific adaptor roles in both the nucleus and cytoplasm (Sakamuro and Prendergast, 1999; Wigge and McMahon, 1998). Nevertheless, there is evidence that the ubiquitously expressed nuclear Bin1 isoform that interacts with c-Myc can modulate transcription mediated by this oncoprotein (Elliott *et al.,* 1999) and can participate in cell death and differentiation decisions that it controls (Prendergast, 1999). Therefore, loss or inactivation of *Bin1* in

cancer may also contribute to the loss of differentiated properties of prostate cells, including their reliance upon androgen and adhesion signals, which occur during malignant progression. Although there is a close association between acquisition of androgen independence and metastatic capability, there is not yet any evidence that Bin1 is regulated by androgens (data not shown). However, Bin1 has been identified also as an integrin-binding protein (Wixler *et al.*, 1999), so it may have a role in integrin signaling to the nucleus. Given opposing trends in the expression of c-Myc and Bin1 in prostate cancer, we favor the notion that Bin1 loss or inactivation may help desensitize cells to apoptosis or differentiation signals, allowing them to amplify c-*Myc* or other oncogenes without apoptotic penalty.

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Bin2, a Functionally Nonredundant Member of the BAR Adaptor Gene Family

Kai Ge*,[1] and George C. Prendergast*,†,[2]

*The Wistar Institute, Philadelphia, Pennsylvania 19104; and †Glenolden Laboratory, DuPont Pharmaceuticals Company, Glenolden, Pennsylvania 19036

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BAR family proteins are a unique class of adaptor proteins characterized by a common N-terminal fold of undetermined function termed the BAR domain. This set of adaptors, which includes the mammalian proteins amphiphysin and Bin1 and the yeast proteins Rvs167p and Rvs161p, has been implicated in diverse cellular processes, including synaptic vesicle endocytosis, actin regulation, differentiation, cell survival, and tumorigenesis. Here we report the identification and characterization of Bin2, a novel protein that contains a BAR domain but that is otherwise structurally dissimilar to other members of the BAR adaptor family. The Bin2 gene is located at chromosome 4q22.1 and is expressed predominantly in hematopoietic cells. Bin2 is upregulated during differentiation of granulocytes, suggesting that it functions in that lineage. Bin2 formed a stable complex in cells with Bin1, but not with amphiphysin, in a BAR domain-dependent manner. This finding indicates that BAR domains have specific preferences for interaction. However, Bin2 did not influence endocytosis in the same manner as brain-specific splice isoforms of Bin1, nor did it exhibit the tumor suppressor properties inherent to ubiquitous splice isoforms of Bin1. Thus, Bin2 appears to encode a nonredundant function in the BAR adaptor gene family.

INTRODUCTION

BAR proteins are a family of adaptor proteins implicated in a diverse set of cellular processes, including cell growth control, cell survival, differentiation, endocytosis, and actin organization. This family is characterized by a common N-terminal domain termed the BAR domain and includes the mammalian Bin1 and amphiphysin proteins and the yeast Rvs167 and Rvs161 proteins. Amphiphysin, the first member of the BAR family to be identified, was named for its biochemical properties rather than for its cellular function (Lichte *et al.,* 1992). Subsequent studies showed that amphiphysin is a cytosolic neuronal protein and functions in synaptic vesicle endocytosis (Wigge and McMahon, 1998). Amphiphysin also acts as a paraneoplastic autoimmune antigen in rare cancers of the breast, lung, and other tissues that are associated clinically with certain nervous system disorders (Antoine *et al.,* 1999; Dropcho, 1996; Folli *et al.,* 1993). Amphiphysin can be detected in breast and certain other nonneuronal tissues (Floyd *et al.,* 1999), but it is expressed predominantly in the central nervous system.

The yeast proteins Rvs167p and Rvs161p were identified in a screen for mutations that caused reduced viability upon nutrient starvation (Bauer *et al.,* 1993; Crouzet *et al.,* 1991). Yeast lacking these proteins can proliferate but cannot properly exit the cell cycle and sporulate when starved, suggesting roles in cell growth regulation. Rvs proteins are cytosolic and have been implicated in actin regulation and endocytosis (Balguerie *et al.,* 1999; Bauer *et al.,* 1993; Breton and Aigle, 1998; Colwill *et al.,* 1999; Lila and Drubin, 1997; Sivadon *et al.,* 1995). Rvs161p has a unique function in cell fusion during mating that can be separated from its role in endocytosis (Brizzio *et al.,* 1998). Rvs167p may have a unique role in linking actin regulation to the cell cycle, insofar as it is an important substrate for Pho85p (Lee *et al.,* 1998), a cell cycle kinase recently defined as the yeast homolog of the mammalian enzyme cdk5 (Huang *et al.,* 1999; Nishizawa *et al.,* 1999).

The mammalian Bin1 gene (bridging integrator-1) encodes a set of BAR proteins with different functions that are generated by tissue-specific splicing. Bin1 was identified initially through its ability to interact with and inhibit the oncogenic properties of the c-Myc oncoprotein (Sakamuro *et al.,* 1996). Subsequent analysis identified two ubiquitous splice isoforms and several tissue-specific splice isoforms expressed predominantly in skeletal muscle and brain (Butler *et al.,* 1997; Ramjaun *et al.,* 1997; Tsutsui *et al.,* 1997; Wechsler-Reya *et al.,* 1997b, 1998). Brain-specific splice isoforms are

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[1] Current address: The Rockefeller University, New York, NY 10012.

[2] To whom correspondence should be addressed. Telephone: (610) 237-7847. Fax: (610) 237-7937. E-mail: george.c.prendergast@dupontpharma.com.

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most closely related to amphiphysin and have been termed alternately amphiphysin II (Butler *et al.,* 1997; Ramjaun *et al.,* 1997; Tsutsui *et al.,* 1997; Wigge *et al.,* 1997; Wigge and McMahon, 1998). However, outside the brain it is clear that Bin1 has unique functions that are not amphiphysin-like. First, ubiquitous isoforms found outside the brain are localized in the nucleus as well as the cytosol, whereas brain-specific isoforms are apparently strictly cytosolic. Second, only brain-specific isoforms include sequences required for interaction with clathrin and elements of the endocytotic machinery (Ramjaun and McPherson, 1998). Although amphiphysin is commonly referred to as the ortholog of Rvs167, Bin1 may fulfill this role, since outside the brain it is closer in size and structure as well as ubiquitous rather than tissue restricted in expression. Nuclear isoforms that associate with c-Myc can suppress c-Myc-dependent gene activation and have been implicated in the control of cell proliferation, differentiation, and programmed cell death (Elliott *et al.,* 1999; Mao *et al.,* 1999; Prendergast, 1999; Sakamuro *et al.,* 1996; Wechsler-Reya *et al.,* 1998). Moreover, there is extensive evidence that c-Myc-interacting isoforms have tumor suppressor properties that are frequently missing or inactivated in human cancers, including malignant melanoma, invasive breast cancer, and metastatic prostate cancer (Ge *et al.,* 1999, 2000a, b). In contrast, amphiphysin and brain-specific splice isoforms of Bin1 lack tumor-suppressor activity. Significantly, one mechanism by which Bin1 is functionally inactivated in human cancer is by missplicing of a neuronal exon (Ge *et al.,* 1999). Thus, it is clear that while BAR adaptor proteins share a common domain they have divergent physiological functions.

In this study, we report the characterization of a novel mammalian BAR adaptor protein termed Bin2 (bridging integrator-2). The expression pattern of Bin2 is reminiscent of amphiphysin in that it appears to be largely tissue restricted, except that in the case of Bin2 it is found predominantly in hematopoietic cells rather than in the central nervous system. Interestingly, Bin2 lacks sequences found in Bin1 or amphiphysin that mediate interaction with c-Myc or with clathrin and the endocytotic machinery. Bin2 also lacks a C-terminal SH3 domain found in the other mammalian BAR proteins, instead including a unique long C-terminal extension. Consistent with these structural differences, Bin2 lacked endocytotic or antiproliferative properties displayed by amphiphysin and Bin1. Thus, Bin2 encodes a novel and nonredundant function in the BAR adaptor gene family.

MATERIALS AND METHODS

Cloning and plasmid vectors. A TBLASTN search of the expressed sequence tag (EST) database with the amino acid sequences encompassing the C-terminal of the BAR domain of Bin1 identified a germinal B cell cDNA (GenBank Accession No. AA452680) that encoded a polypeptide related to but nonidentical with amphiphysin or Bin1. Using this EST cDNA as a probe, a full-length cDNA was obtained from a human leukocyte 5′-Stretch Plus cDNA library (HL5019t; Clontech). The complete DNA sequence of this clone was determined by standard methods (GenBank Accession No. AF146531). Human genomic Bin2 BAC clones, used for chromosome mapping experiments, were isolated by Genome Systems, Inc., using the Bin2 cDNA fragment. An epitope-tagged Bin2 cDNA was engineered by the replacement of the initiator methionine with the influenza virus hemagglutinin (HA) epitope recognized by the monoclonal antibody 12CA5 (Niman *et al.,* 1983). For *in vivo* expression Bin2 and HA-Bin2 were subcloned into the mammalian vector pcDNA3.1/neo (Invitrogen). A similar vector for expression of full-length Bin1 has been described (Sakamuro *et al.,* 1996; Wechsler-Reya *et al.,* 1998). Full-length cDNA for Bin1−10+12ABCD [also known as amphiphysin IIa (Ramjaun *et al.,* 1997)] or human amphiphysin was also subcloned into pcDNA3.1/neo for expression. BAR deletion mutants of Bin1 that lack the amino acid residues indicated were constructed as described (Elliott *et al.,* 1999) and subcloned for expression into pcDNA3.1/neo.

Fluorescence in situ hybridization (FISH) chromosome analysis. FISH was performed using a Bin2 genomic BAC clone labeled by nick-translation with digoxigenin–dUTP and metaphase chromosomes isolated from PHA-stimulated normal peripheral blood lymphocytes. A biotin-labeled probe specific for the centromere of chromosome 4 was also included in the hybridization. Slides were developed using fluoresceinated anti-digoxigenin antibodies and Texas red avidin. Two experiments were done and a total of 80 metaphase cells were analyzed with 73 exhibiting specific labeling. Map location was determined on chromosome 4 by measuring the physical distance on 10 labeled chromosomes, computed to be 27% of the distance from the centromere to the telomere of chromosome arm 4q, an area that corresponds to band 4q22.1.

Blot analyses. Genomic DNA was isolated and analyzed by Southern analysis by standard methods (Sambrook *et al.,* 1989) using full-length Bin2 cDNA as a hybridization probe. Total cytoplasmic RNA was prepared from adherent cell lines as described (Prendergast and Cole, 1989). Total RNA from suspension cell lines was isolated using a commercial kit employing TriZol reagent (Life Technologies). Fifteen micrograms of RNA per lane was fractionated on formaldehyde gels and analyzed essentially as described (Prendergast and Cole, 1989), using Bin2 cDNA as a hybridization probe.

Protein–protein interaction. Bin2 or HA-Bin2 was cotranslated *in vitro* with Bin1 or amphiphysin proteins using a commercial reticulocyte extract kit (Promega). Complex formation was assessed by immunoprecipitation with anti-HA antibody 12CA5 (BMB), anti-amphiphysin (Transduction Laboratory), or anti-Bin1 antibody 99D (Wechsler-Reya *et al.,* 1997a), followed by SDS–PAGE and fluorography. For *in vivo* experiments, 293T cells were transfected with vectors for Bin1, HA-Bin2, or no insert using LipofectAmine 2000 following the vendor's protocol (Life Technologies). Forty-eight to sixty h after transfection, cells were collected and lysed in ice-cold NP-40 lysis buffer [150 mM NaCl, 0.5% NP-40, 50 mM Tris–Cl (pH 8.0), 1 mM PMSF, 1 μg/ml leupeptin, 0.4 U/ml aprotinin, 10 μg/ml leupeptin]. Cell lysates were immunoprecipitated with Bin1 mAb 99D or anti-HA mAb 12CA5 and immunoblotted with 99D or the polyclonal anti-HA antibody sc-805 (Santa Cruz Biotechnology).

Tissue culture. Human tumor cell lines, 293T cells (ATCC CRL-1573), and COS7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Life Technologies) and 50 U/ml penicillin and streptomycin (Fisher). The human myeloid leukemia cell line HL60 was maintained in RPMI 1640 supplemented with 15% FCS (Life Technologies) and 50 U/ml penicillin and streptomycin (Fisher).

Endocytosis assay. COS cells were plated on coverslips and transfected 24 h later with expression vectors for Bin2 or the Bin1 brain-specific splice isoform Bin1−10+12ABCD [also known as amphiphysin IIa (Ramjaun *et al.,* 1997)]. Bin1−10+12ABCD has been shown to inhibit transferrin uptake in COS cells (Wigge *et al.,* 1997). A modified calcium phosphate protocol was used for transfection

A

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MAEGKAGGAAGLFAKQVQKKFSRAQEKVLQKLGKAVETKDERFEQSASNFYQQQAEGHKLYKDLKNFLSAVKVMHESSKRVSET
LQEIYSSEWDGHEELKAIVWNNDLLWEDYEEKLADQAVRTMEIYVAQFSEIKERIAKRGRKLVDYDSARHHLEAVQNAKKDEAK
TAKAEEEFNKAQTVFEDLNQELLEELPILYNSRIGCYVTIFQNISNLRDVFYREMSKLNHNLYEVMSKLEKQHSNKVFVVKGLS
SSSRRSLVISPPVRTATVSSPLTSPTSPSTLSLKSESESVSATEDLAPDAAQGEDNSEIKELLEEEEIEKEGSEASSSEEDEPL
PACNGPAQAQPSPTTERAKSQEEVLPSSTTPSPGGALSPSGQPSSSATEVVLRTRTASEGSEQPKKRASIQRTSAPPSRPPPPR
ATASPRPSSGNIPSSPTASGGGSPTSPRASLGTGTASPRTSLEVSPNPEPPEKPVRTPEAKENENIHNQNPEELCTSPTLMTSQ
VASEPGEAKKMEDKEKDNKLISADSSEGQDQLQVSMVPENNNLTAPEPQEEVSTSENPQL
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B

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Bin2      1   MAE-GKAGGAAGLFAKQVQKKFSRAQEKVLQKLGKAVETKDERFEQSASNFYQQQAEGHK    59
              MAE G  G  AG  A  VQKK +RAQEKVLQKLGKA ETKDE+FEQ   NF +Q +EG +
Bin1      1   MAEMGSKGVTAGKIASNVQKKLTRAQEKVLQKLGKADETKDEQFEQCVQNFNKQLTEGTR    60
              MA         G+FAK VQK+  RAQEKVLQKLGKA ETKDE FE    NF  Q AEG +
Amphi     1   MADIK-----TGIFAKNVQKRLNRAQEKVLQKLGKADETKDEQFEEYVQNFKRQEAEGTR    55

Bin2     60   LYKDLKNFLSAVKVMHESSKRVSETLQEIYSSEWDGHEELKAIVWNNDLLWEDYEEKLAD   119
              L KDL+ +L++VK MHE+SK+++E LQE+Y  +W G +E   I  NNDLLW DY +KL D
Bin1     61   LQKDLRTYLASVKAMHEASKKLNECLQEVYEPDWPGRDEANKIAENNDLLWMDYHQKLVD   120
              LQ++L  +L+A+K M+E+S ++ E L E+Y  +W G E+   +    D+LWED+  KL D
Amphi    56   LQRELG-YLAAIKGMQEASMKLTESLHEVYEPDWYGREDVKMVGEDCDVLWEDFHQKLVD   115

Bin2    120   QAVRTMEIYVAQFSEIKERIAKRGRKLVDYDSARHHLEAVQNA-KKDEAKTAKAEEEFNK   178
              QA+ TM+ Y+ QF +IK RIAKRGRKLVDYDSARHH E++Q A KKDEAK AKAEEE  K
Bin1    121   QALLTMDTYLGQFPDIKSRIAKRGRKLVDYDSARHHYESLQTAKKKDEAKIAKAEEELIK   180
               ++ T + Y+ QF +IK RIAKR RKLVDYDSARHHLE+LQ++ +KDE++ +KAEEEF K
Amphi   116   GSLLTLDTYLGQFPDIKNRIAKRSRKLVDYDSARHHLEALQSSKRKDESRISKAEEEFQK   176

Bin2    179   AQTVFEDLNQELLEELPILYNSRIGCYVTIFQNISNLRDVFYREMSKLNHNLYEVMSKLE   238
              AQ VFE++N +L EELP L+NSR+G YV  FQ+I+ L + F++EMSKLN NL +V+  LE
Bin1    181   AQKVFEEMNVDLQEELPSLWNSRVGFYVNTFQSIAGLEENFHKEMSKLNQNLNDVLVGLE   240
              AQ VFE+ N +L EELP L+ SR+G YV  F N+S L   F++E + L H LYEVM+KL
Amphi   177   AQKVFEEFNVDLQEELPSLWSRRVGFYVNTFKNVSSLEAKFHKEIAVLCHKLYEVMTKLG   237

Bin2    239   KQHSNKVFVVKG
              KQH +  F VK
Bin1    241   KQHGSNTFTVKA
             +QH  K F + G
Amphi   238  DQHADKAFTIQG
```

FIG. 1. Structure of human Bin2 and its relationship to the BAR family of adaptor proteins. (**A**) Conceptual translation of full-length human Bin2 cDNA isolated from a leukocyte library. (**B**) Alignment of the BAR domains of Bin2, Bin1, and amphiphysin. The BLAST2 algorithm was used for alignment; amphiphysin was aligned manually. Identical or similar residues (the latter marked by a plus sign) are indicated relative to the Bin2 sequence. (**C**) BAR family adaptor proteins. The cartoon compares the domain structure of Bin2 with known BAR proteins in mammals (Bin1 and amphiphysin) and the yeast *Saccharomyces cerevisiae* (Rvs167p and Rvs161p). Different splice isoforms of Bin1 are noted; exon numbers are from Wechsler-Reya *et al.* (1997b).

(Chen and Okayama, 1987). Thirty h after transfection, cells were incubated with FITC-conjugated transferrin (Molecular Probes), and transferrin receptor-mediated endocytosis was monitored by indirect immunofluorescence as described (Benmarah *et al.,* 1999). Cells expressing HA-Bin2 or Bin1−10+12ABCD were identified with monoclonal antibodies anti-HA 12CA5 or anti-Bin1 99D, respectively (Niman *et al.,* 1983; Wechsler-Reya *et al.,* 1997a). A Texas red-conjugated horse anti-mouse antibody (Vector) was used to visualize the primary antibody.

RESULTS

Bin2 is a mammalian BAR adaptor protein that lacks an SH3 domain. We observed previously that a polyclonal Bin1 antiserum crossreacted with non-Bin1 polypeptides in cells that do not express amphiphysin (Sakamuro *et al.,* 1996), suggesting that additional BAR family adaptor proteins existed. Subsequent epitope mapping of this antiserum defined a major epitope at the extreme C-terminus of the BAR domain (Wechsler-Reya *et al.,* 1997a). A TBLASTN search of the EST database with amino acid sequences derived from this region identified a partial germinal B cell cDNA (GenBank Accession No. AA452680) that encoded a polypeptide related to but nonidentical to amphiphysin and Bin1. This cDNA was used as a probe to isolate full-length cDNA clones from a human leukocyte phage library. DNA sequence analysis identified a long open reading frame of 564 aa rich in serine and glutamic acid that has a predicted molecular mass of 61709 Da (see Fig. 1A). Southern analysis using the cDNA probe confirmed the presence of Bin2 sequences in human genomic DNA (data not shown). Comparison of Bin1 to the polypeptide encoded by this cDNA, which was termed Bin2, confirmed the presence of a complete BAR motif that was 61% identical and 75% similar to Bin1 with boundaries of aa 1–249 in Bin2 and aa 1–251 in Bin1 (see Fig. 1B). Bin2 was identical to Bin1 within the region of the BAR domain that is most highly conserved in evolution (aa 138–155). Amphiphysin has a nonidentical residue in this region and was slightly less similar overall as computed by the BLAST algorithm. A second segment of the BAR domain that was

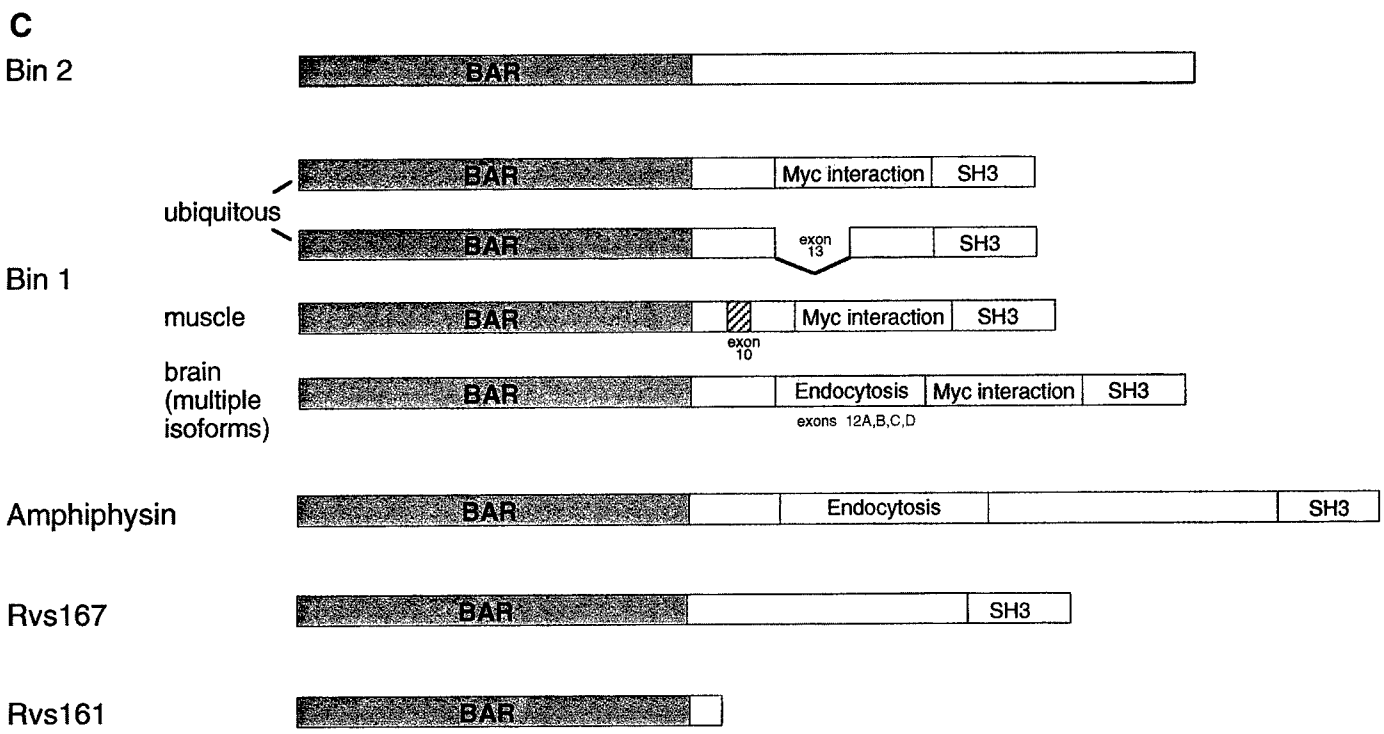

FIG. 1—*Continued*

highly conserved in these proteins was aa 23–45, which encompasses a region predicted to be helical in configuration (data not shown). The high degree of conservation between the N-terminal region of Bin2 and the BAR domains of Bin1 and amphiphysin identified Bin2 as a third member of the BAR family to be identified in mammalian cells.

Outside the BAR domain Bin2 lacked any similarity to other members of the BAR adaptor family (see Fig. 1C). The C-terminal regions in this family exhibit considerable structural diversity, especially in the case of Bin1, which is alternately spliced constitutively or in a tissue-specific manner. The C-terminal extension in Bin2 (aa 250–564) included acidic and serine/proline-rich segments but was otherwise unrelated to either BAR family adaptors or other known proteins. One possible exception was a distant relationship noted by database comparisons using the BLAST algorithm between the midsection of Bin2 (aa 179–336) and a central region of Daxx, a ubiquitous protein implicated in a variety of nuclear processes connected to transcription and programmed cell death (Chang *et al.,* 1998; Michaelson *et al.,* 1999; Torii *et al.,* 1999). The *E* value computed for the BLAST sequence alignment (which measures its relative significance) was higher than the analogous alignment between Bin2 and Rvs161 (9×10^{-3} versus 7.6×10^{-2}, respectively). Nevertheless, since the score was relatively low the significance of this relationship was uncertain. As noted above, Bin2 lacked a C-terminal SH3 domain that is found in all BAR adaptor proteins except Rvs161 (Fig. 1C). However, while Rvs161 lacks an SH3 it also lacks a C-terminal extension. Thus, Bin2 seems unlikely to be

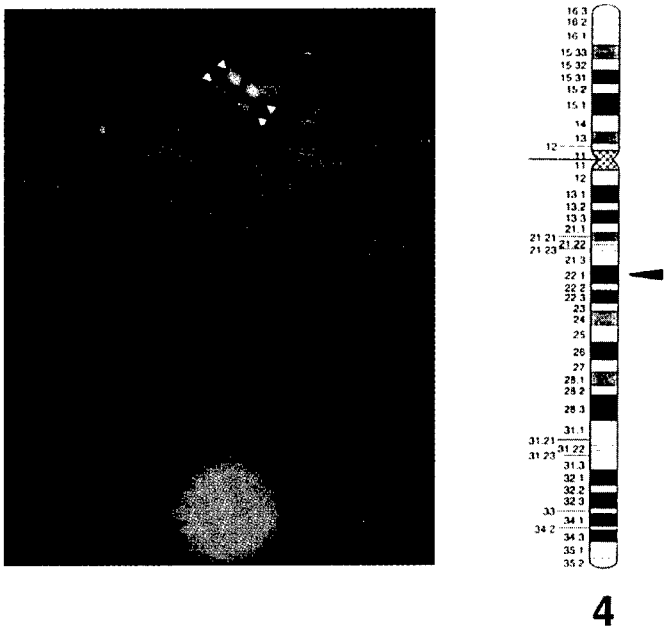

FIG. 2. The human Bin2 gene localizes to chromosome 4q22.1. Chromosomal location of Bin2 was mapped by FISH of metaphase chromosomes isolated from PHA-stimulated normal peripheral blood lymphocytes. Probes were a Bin2 genomic BAC clone labeled with digoxigenin–dUTP and a biotin-labeled DNA fragment that is specific for the centromere of chromosome 4. Slides were developed with fluoresceinated anti-digoxigenin antibodies and Texas red avidin. The white arrowheads indicate a representative pattern hybridization of 73 metaphase chromosome spreads analyzed, with red color identifying the chromosome 4 centromere and green color identifying the Bin2 locus on the long arm of the chromosome. The right side presents the map location of Bin2 as computed by determining the average physical distance between labeled loci on 10 separate chromosomes, an area that corresponds to band 4q22.1.

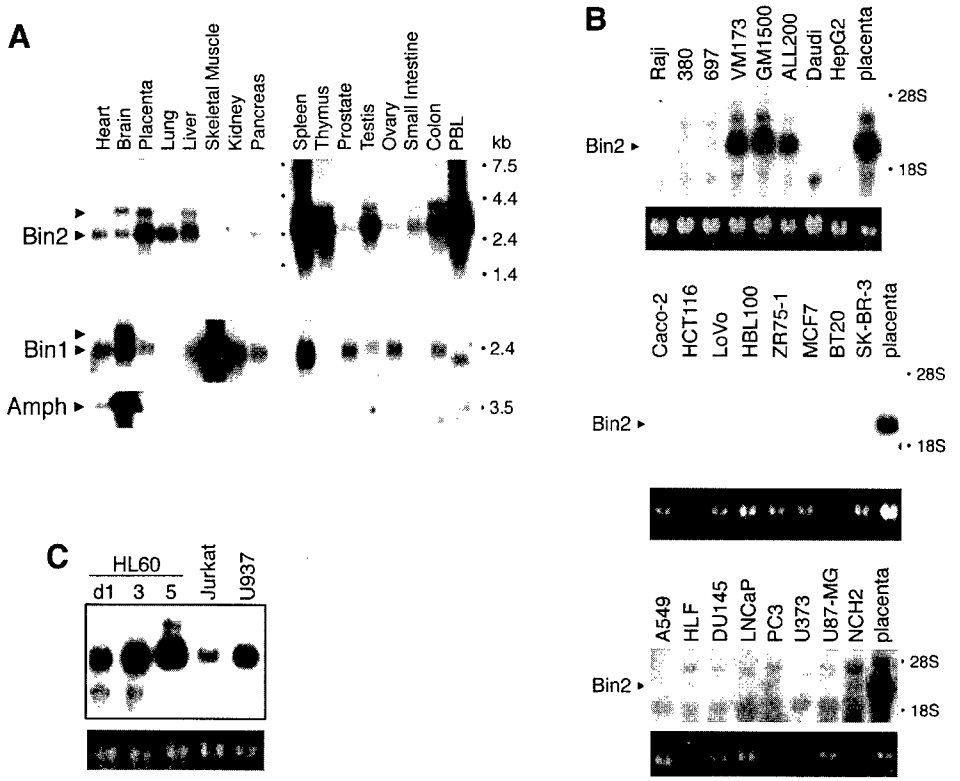

FIG. 3. Bin2 is expressed predominantly in hematopoietic cells and is induced during granulocytic differentiation. RNAs isolated from the tissues and cell lines indicated were subjected to Northern analysis using Bin2 cDNA as a hybridization probe. Ethidium bromide-stained gels are provided as RNA loading controls. (**A**) Human tissues. Blots were stripped and rehybridized sequentially to Bin1 and amphiphysin cDNA probes to contrast the patterns of expression of mammalian BAR proteins. An apparent splice isoform(s) of Bin2 is indicated by the upper arrowhead in the top blot. The several major splice isoforms of Bin1 seen specifically in brain (Wechsler-Reya *et al.,* 1997b) are indicated by the upper arrowhead in the middle blot. A ubiquitous splice isoform of Bin1 (second ubiquitous form shown in Fig. 1C) predominates in hematopoietic cells and can be seen in spleen, thymus, and peripheral blood lymphocytes (PBL). Amphiphysin expression is essentially confined to brain. (**B**) Human cell lines. Placenta and HepG2 hepatoma cells are positive and negative controls for expression, respectively. Top: lymphoid cell lines. Middle and bottom: adherent cell lines derived from breast, colon, lung, liver, prostate, glial cell, and astrocyte. (**C**) Bin2 is induced during granulocytic differentiation. HL60 promyelocytic leukemia cells were treated for the times indicated with DMSO to induce granulocytic differentiation, and RNA was isolated for Northern analysis with Bin2 cDNA. Jurkat is a T lymphoid cell line and U937 is a myeloid cell line to compare expression in other positive blood cell lines.

the mammalian ortholog of Rvs161 but instead a gene that arose later in evolution. Bin2 also lacked sequences required in Bin1 for interaction with c-Myc (Elliott *et al.,* 1999; Sakamuro *et al.,* 1996) or in amphiphysin or brain-specific splice isoforms of Bin1 for interaction with clathrin and other elements of the endocytotic machinery (Ramjaun and McPherson, 1998; P. de Camilli, New Haven, CT, pers. comm., 10 Jan. 1999). Figure 1C summarizes the structural features of BAR proteins. Taken together, the structural differences between Bin2 and other members of the BAR adaptor family suggested that Bin2 encoded a nonredundant function. We concluded that Bin2 was a novel mammalian BAR protein likely to play a unique role in cells.

The human Bin2 gene is located on chromosome 4q22.1 and exhibits aberrant organization in hepatoma cells. The Bin2 cDNA was used to isolate three human genomic BAC clones by standard methods. Restriction analysis and Southern hybridization of these clones and comparison to genomic Southern hybridizations confirmed the presence of Bin2 sequences and ruled out the possibility that a pseudogene was cloned. One of the clones, F727, was used to perform FISH analysis of metaphase chromosomes isolated from normal peripheral blood lymphocytes. Specific hybridization signals were detected on the long arm of a group B chromosome consistent with chromosome 4 on the basis of size, morphology, and banding pattern. An experiment that included a second probe specific for the centromere sequences of chromosome 4 confirmed this interpretation. A total of 80 metaphase cells were analyzed, with 73 exhibiting specific labeling (see Fig. 2). Measurements of 10 specifically labeled chromosomes 4 demonstrated that the Bin2-specific hybridization signal was located at a position 27% of the distance from the centromere to the telomere of chromosome arm 4q, an area corresponding to band 4q22.1.

Bin2 is expressed predominantly in hematopoietic cells and is upregulated during monocytic differentiation. Northern analyses of human tissues and cell lines were performed to investigate the range of expression of Bin2 and to compare it with amphiphysin and Bin1 expression (see Fig. 3). The major message hybridized was ~2.6 kb, which was in reasonable

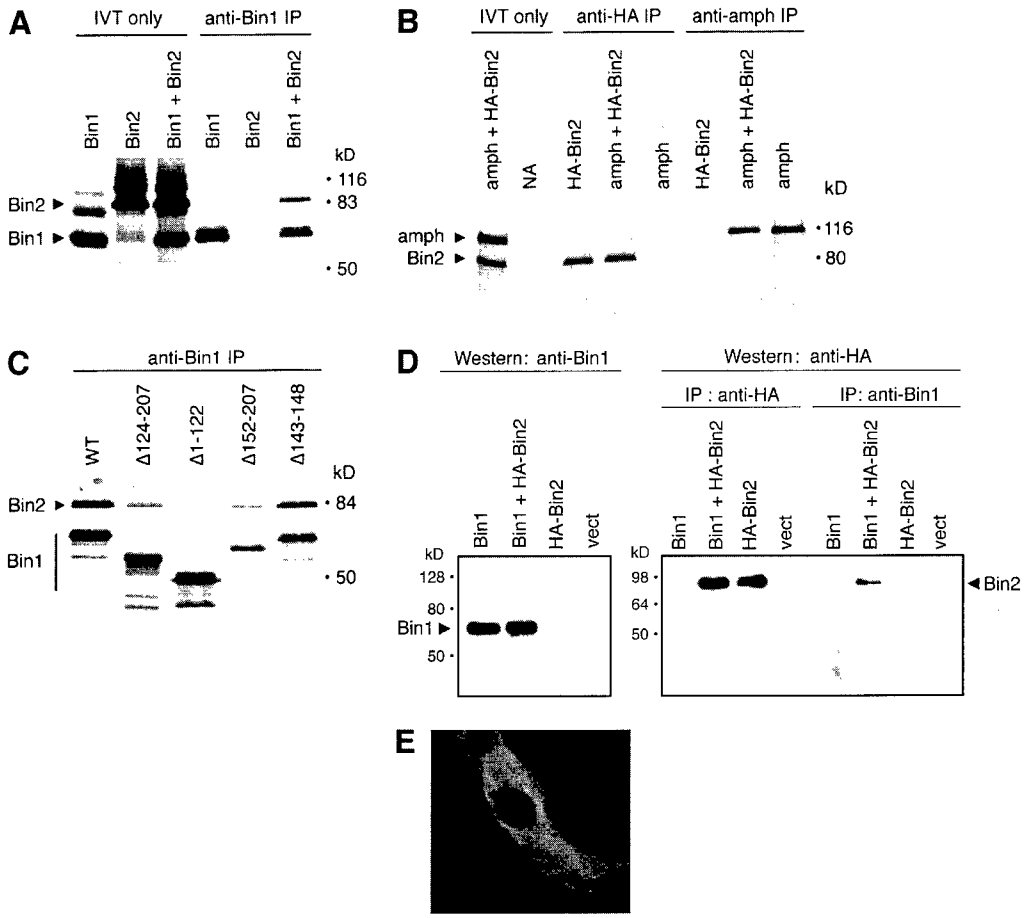

FIG. 4. Bin2 forms a stable BAR domain-dependent complex with Bin1 but not with amphiphysin. (**A**) Bin2 associates stably with Bin1. Bin2 and Bin1 cDNAs were subjected to *in vitro* translation with empty vector or with each other in the presence of [^{35}S]methionine. Two microliters of IVT products was fractionated directly on SDS–PAGE (IVT only); 10 μl of IVT products was subjected to immunoprecipitation with anti-Bin1 antibody 99D (Wechsler-Reya *et al.,* 1997a) before SDS–PAGE fractionation and fluorography. (**B**) Bin2 does not interact with amphiphysin. HA-Bin2 and amphiphysin cDNAs were subjected to *in vitro* translation and immunoprecipitation as above, using anti-HA 12CA5 or anti-amphiphysin. (**C**) N-terminal sequences in the Bin1 BAR domain are crucial for association with Bin2. A set of Bin1 deletion mutants lacking the amino acid residues indicated (Sakamuro *et al.,* 1996) was cotranslated with Bin2 and subjected to immunoprecipitation, SDS–PAGE, and fluorography as above. (**D**) Bin2 and Bin1 associate *in vivo.* COS cells were transfected with expression vectors for HA-Bin2 or Bin1 or with no insert. Cell extracts were prepared 2 days later and subjected to IP–Western analysis with anti-Bin1 and anti-HA antibodies. The left shows the results of straight Western analysis with anti-Bin1. The right shows Western analysis using anti-HA antibody of immunoprecipitates generated by either anti-HA or anti-Bin1. (**E**) Bin2 is cytosolic. COS cells were transfected as above with HA-Bin2 and processed for indirect immunofluorescence as described (Wechsler-Reya *et al.,* 1998), using anti-HA antibody to identify Bin2 and Texas red-conjugated anti-mouse IgG to visualize the antigen.

agreement with the ~2.2-kb cDNA characterized, but an additional message of ~3.5 kb was expressed at lower levels in several tissues (see Fig. 3A, top). The hybridization stringency in the experiment was high, suggesting that these messages were derived by alternate splicing, as seen in Bin1 (see Fig. 3A, middle), but a distinct isoform was not ruled out. Notably, steady-state RNA levels were at least two magnitudes higher in spleen and peripheral blood leukocytes than in other tissues and significantly higher in thymus, colon, and placenta than in other tissues. This pattern of expression suggested that Bin2 might be preferentially expressed in hematopoietic cells (colon and placenta are comparatively rich in lymphocytes and monocytes, respectively). The pattern of Bin2 expression contrasted with Bin1, which was ubiquitous, with highest expression in brain and muscle, and with amphiphysin, which was confined essentially to brain (see Fig. 3A, bottom).

Additional experiments supported the notion that Bin2 was expressed predominantly in blood cells and that its presence in most tissues was a consequence of its high expression in hematopoietic cells that are present in tissue isolates. First, Bin2 was undetectable by Northern analysis of 21 human cell lines derived from a variety of tissues, including breast, lung, prostate, brain, connective tissue (fibroblast), liver, and colon, despite detection of Bin2 in these tissues. In contrast, Bin2 message was strongly expressed in several human lymphoid and lymphoid cell lines, including GM1500, ALL200, BV173, and HL60 (see Fig. 3B). Second, "virtual" analyses performed by comparing

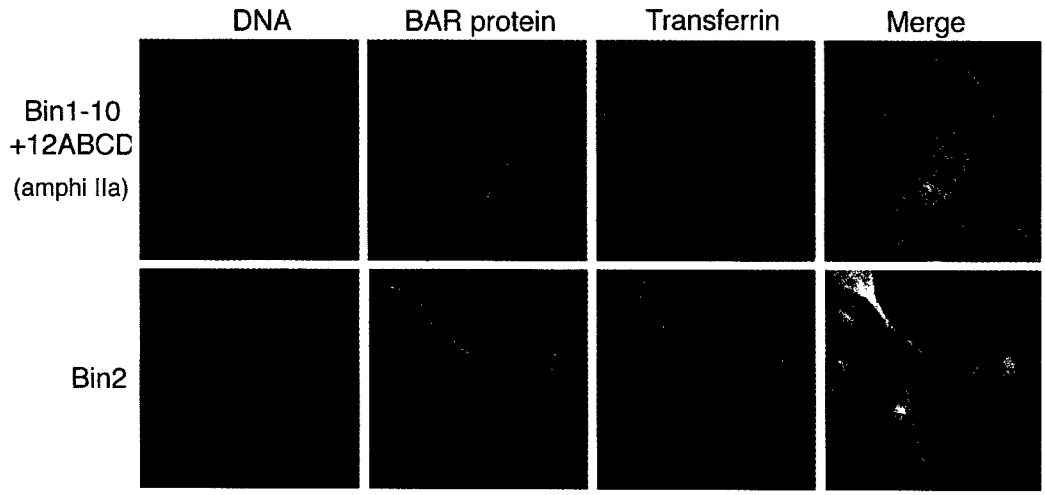

FIG. 5. Bin2 does not inhibit receptor-mediated endocytosis. Transferrin uptake as a measurement of receptor-mediated endocytosis was assayed essentially as described (Benmerah *et al.,* 1999). Briefly, COS cells were transfected with vectors for Bin1−10+12ABCD (amphiphysin IIa) or HA-Bin2 and incubated 24 h later with fluorescein-conjugated transferrin. All cells were stained with DAPI to visualize DNA. Bin1−10+12ABCD and Bin2 were detected with 99D (1:100 v/v hybridoma supernatant) or anti-HA 12CA5 (4 μg/ml), respectively, using a Texas red-conjugated secondary antibody (1:200 v/v) to visualize the proteins for immunofluorescence microscopy. Images were collected and processed using a confocal microscope apparatus.

Bin2 sequences to the EST database, which provides information about the tissue source for cDNA libraries (which enrich for tissue-specific messages), offered an additional line of confirmation that Bin2 is expressed mainly in blood cells (data not shown). The likelihood that the lack of expression in clonal cell lines was due to *in vitro* selection against antiproliferative activity was ruled out (see below). In summary, the results argued that Bin2 was expressed predominantly in blood cells.

Consistent with a functional role in blood lineages, we observed that Bin2 was induced during granulocytic differentiation of HL60 cells, a promyelocytic leukemia cell line. HL60 cells are induced to differentiate to granulocytes by treatment with dimethyl sulfoxide (DMSO). Northern analysis demonstrated increased steady-state levels of Bin2 RNA after 5 days of DMSO treatment relative to early times or to control Jurkat T cells or U937 myeloid cells (see Fig. 3C). The elevation of Bin2 during myeloid differentiation was reminiscent of a similar elevation of Bin1 that occurs during myoblast differentiation (Mao *et al.,* 1999; Wechsler-Reya *et al.,* 1998). We concluded that Bin2 was expressed predominantly in hematopoietic cells and that it was likely to function in granulocytes and other blood cells.

Bin2 is a cytosolic protein that associates with Bin1, but not amphiphysin, in a BAR-dependent manner. Rvs161 and Rvs167 form complexes in yeast, and amphiphysin and Bin1 form stable complexes in brain extracts (Navarro *et al.,* 1997; Wigge *et al.,* 1997). We therefore examined the ability of Bin2 to interact with Bin1 or amphiphysin *in vitro* and *in vivo.* Bin2 cDNA was subjected to *in vitro* translation (IVT) in the presence or absence of Bin1 or amphiphysin and complex formation was assessed by coimmunoprecipitation (IP) with anti-Bin1 antibody 99D (Wechsler-Reya *et al.,* 1997a) or an anti-amphiphysin antibody, followed by SDS–PAGE and fluorography. For some experiments, a derivative of Bin2 was constructed that included an N-terminal influenza HA epitope tag that is recognized by the monoclonal antibody 12CA5 (Niman *et al.,* 1983). Expression of this HA-tagged derivative, termed HA-Bin2, was confirmed by DNA sequencing and by *in vitro* translation (data not shown). Bin2 migrated with an apparent mobility of ~80 kDa, greater than the predicted molecular mass of ~61 kDa but reminiscent of the similar aberrant mobility displayed by Bin1 on Laemmli gels (Sakamuro *et al.,* 1996).

Interestingly, Bin2 formed a stable complex with Bin1 but not with amphiphysin. Interaction with Bin1 was consistent with evidence that BAR proteins form oligomers (Navarro *et al.,* 1997; Wigge *et al.,* 1997). The Bin2–Bin1 complex was stable under the conditions used and was approximately equimolar (see Fig. 4A). Notably, under the same conditions Bin2 and amphiphysin did not form stable complexes (see Fig. 4B). This result suggested either that these proteins could not form a complex or that their interaction was relatively unstable. Using a set of Bin1 deletion mutants (Elliott *et al.,* 1999), we mapped the determinants needed for interaction with Bin2. Deletion of the so-called unique central region (white boxes in Fig. 1C), the Myc-binding domain, or the SH3 domain had no effect on the efficiency of Bin2 co-IP (data not shown). In contrast, deletions within the BAR domain reduced or abolished interaction (see Fig. 4C). A major requirement for interaction mapped to the N-terminal region of Bin1 (aa 1–122), which includes one of the highly conserved segments of the BAR domain (see Fig. 1B). In contrast, deletion of sequences comprising the C-terminal half of the BAR domain (aa 124–207) reduced association only about twofold. A second deletion within this region (aa 152–207) produced a similar effect, consistent with some contribution of this region

to the efficiency of Bin2–Bin1 interaction. In contrast, deletion of a short segment within the most highly conserved part of the BAR domain (aa 143–148) did not affect interaction at all, supporting the notion that these sequences have a different function (Elliott *et al.,* 1999). In summary, the results indicated that N-terminal BAR sequences in Bin1 were crucial for specific complex formation with Bin1 and that conserved C-terminal BAR sequences had a lesser or different role.

To confirm that Bin1 and Bin2 could associate stably *in vivo* we conducted a similar set of IP experiments from extracts derived from COS cells transiently transfected with mammalian expression vectors. COS cells were transfected with Bin1 and HA-Bin2 vectors and then processed for IP–Western analysis by standard methods. HA-Bin2 was observed specifically in Bin1 immunoprecipitates (see Fig. 4D). Similar results were obtained by substituting in the experiment the brain-specific isoform Bin1−10+12ABCD, arguing that different splice isoforms of Bin1 that included identical BAR domains could associate with Bin2 similarly (data not shown). Bin1 splice isoforms vary in their subcellular localization, so we examined the localization of Bin2 in COS cells transfected with HA-Bin2 and processed for indirect immunofluorescence with anti-HA antibody. Bin2 was localized exclusively in the cytosol (see Fig. 4E). We concluded that Bin2 was a cytosolic protein that associated stably with Bin1 in cells in a BAR domain-dependent manner.

Bin2 does not influence receptor-mediated endocytosis. Brain-specific splice isoforms of Bin1 inhibit receptor-mediated endocytosis when overexpressed in COS cells (Wigge *et al.,* 1997). To determine whether Bin2 functions similarly, we compared its ability to inhibit transferrin uptake relative to the brain-specific isoform Bin1−10+12ABCD. Briefly, COS cells were transfected with HA-Bin2 or Bin1−10+12ABCD, incubated 24 h later with fluorescein-conjugated transferrin, and processed for indirect immunofluorescence as described (Benmarah *et al.,* 1999). Cells were stained with DAPI, to locate cell nuclei, and anti-HA or 99D, to locate cells expressing HA-Bin2 or Bin1−10+12ABCD, respectively.

We observed no effect of Bin2 overexpression on transferrin endocytosis in this model (see Fig. 5). Bin1−10+12ABCD markedly inhibited transferrin uptake, as indicated by greatly reduced fluorescein signal in transfected cells. In contrast, untransfected cells in the same microscope field were brightly stained, illustrating high background levels of transferrin endocytosis under these conditions. Bin2 lacked similar activity. Cells transfected with HA-Bin2 exhibited similar levels of fluorescein signal, relative to untransfected cells in the same microscope field. In addition, merged fields showed yellow signal, illustrating overlap of green and red signals for transferrin and Bin2 protein, respectively, confirming that Bin2 did not inhibit transferrin uptake (compare Merge, Fig. 5). We concluded that Bin2 does not function similarly to Bin1 in receptor-mediated endocytosis.

Bin2 lacks antiproliferative activity and does not affect the tumor-suppressor properties of Bin1. Bin1 has tumor-suppressor properties in malignant cells (Elliott *et al.,* 1999; Ge *et al.,* 1999, 2000a, b; Sakamuro *et al.,* 1996), so we investigated whether Bin2 may have similar effects and/or whether it could influence the growth-inhibitory activity of Bin1. For these experiments we employed several malignant cell lines that were susceptible to Bin1 suppression and that lack Bin2 expression (thereby offering a useful null background), including HepG2 hepatoma, MCF-7 breast, A549 lung, and DU145 and PC3 prostate cells. Cells were transfected with the same expression vector used above, which carries a neomycin-resistance cassette, and stably transformed cells were selected in growth medium containing G418. Unlike the case with Bin1 (Sakamuro *et al.,* 1996), we observed little if any effect on colony formation efficiency relative to empty vector. HepG2 exhibited an approximately twofold suppression relative to A549 and other cell lines tested, which showed no difference in colony formation efficiency (see Fig. 6A). To determine whether the apparent suppression of HepG2 reflected growth inhibition, we cloned and expanded a set of G418-resistant HepG2 colonies derived from Bin2 transfection and determined whether the cells stably expressed Bin2. Northern analysis indicated robust expression of Bin2 in all independent cell lines examined (see Fig. 6B). Moreover, we did not see any detrimental effect on cell proliferation (data not shown). Thus, Bin2 expression was compatible with *in vitro* proliferation of all the malignant cells tested, including HepG2. To investigate the possible effects of Bin2 on growth suppression by Bin1, we performed a similar set of colony formation experiments in HepG2 cells, except that cells were cotransfected with untagged empty vector or Bin2 vector and a neomycin-resistance gene-tagged Bin1 vector. Bin1 suppressed colony formation, consistent with previous observations (Elliott *et al.,* 1999; Sakamuro *et al.,* 1996), and cotransfection of Bin2 did not markedly influence this effect (see Fig. 6C). We concluded that Bin2 lacked the tumor-suppressor features inherent to Bin1.

DISCUSSION

This study reports the molecular cloning and characterization of Bin2, the third mammalian BAR protein to be identified. The structure, expression patterns, and preliminary functional analysis of Bin2 each point to a nonredundant function for this protein in cells. Bin2 lacked features seen in the mammalian relatives Bin1 and amphiphysin, which have been implicated in certain signal transduction events related, respectively, to c-Myc and c-Abl in the nucleus (Elliott *et al.,* 1999; Kadlec and Pendergast, 1997; Sakamuro *et al.,* 1996) and to endocytosis in the cytosol (Butler *et*

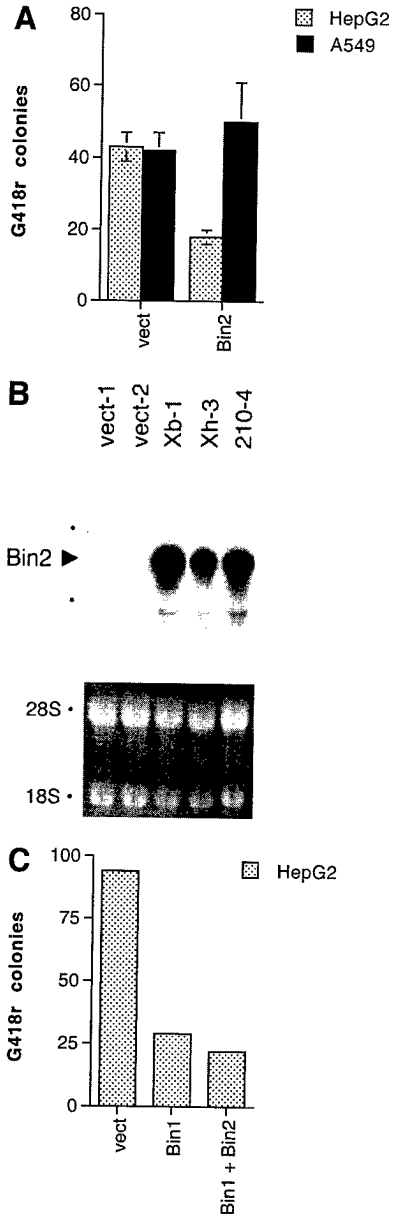

FIG. 6. Bin2 lacks antiproliferative activity and does not affect the tumor suppressor properties of Bin1. (**A**) Colony formation assay. HepG2 hepatoma or A549 lung carcinoma cells were transfected with expression vectors containing a neomycin-resistance gene cassette and Bin2 cDNA or no insert. Stable transformants were selected by culturing cells in G418. Colonies were scored ~3 weeks later by methanol fixation and crystal violet staining and were counted. The results depict the means and standard errors from three trials. (**B**) Northern analysis of HepG2 cell lines derived from transfection with Bin2 or empty vectors. Stably transformed colonies were ring-cloned and expanded into cell lines. RNA was isolated from cells and subjected to Northern analysis using Bin2 cDNA as a hybridization probe. The bottom shows the ethidium bromide-stained gel before transfer as a loading control. (**C**) Bin2 does not affect the tumor suppressor activity of Bin1. Colony formation assay was performed as above. Briefly, HepG2 cells were cotransfected with the expression vectors indicated, and stably transformed colonies were selected in G418.

al., 1997; Ramjaun and McPherson, 1998; Ramjaun *et al.,* 1997; Wigge *et al.,* 1997; Wigge and McMahon, 1998). Bin2 is the first mammalian BAR protein found that lacks an SH3 domain. However, although this is also a feature of the yeast Rvs161 protein, the presence of a C-terminal extension in Bin2 which the yeast protein lacks argues that these proteins are not homologous. Bin2 sequences did not give clear insight into its function. A distant similarity was seen between the central domains of Bin2 and Daxx, but the significance of this relationship is uncertain. Both Daxx and certain Bin1 isoforms appear to influence cell survival decisions at some level but the mechanisms involved are currently obscure (Elliott *et al.,* 2000; Prendergast, 1999). Daxx has a physiological role in the control of programmed cell death (Michaelson *et al.,* 1999) and it interacts with proteins that influence death decisions, such as the Jun kinase regulator apoptosis-signaling kinase-1 and the tumor-suppressor Pml (Chang *et al.,* 1998; Quignon *et al.,* 1998; Torii *et al.,* 1999; Wang *et al.,* 1998). Similarly, ectopic complementation of the frequent deficiencies of Bin1 in cancer cells leads to engagement of a programmed cell death process (Ge *et al.,* 1999, 2000a). We did not observe any effects of Bin2 on cell survival, but further investigation to assess this connection may be worthwhile, insofar as it would be consistent with the role of certain Bin1 isoforms in cancer cells as well as with the role of Rvs proteins in yeast survival under stress conditions.

The human Bin2 gene mapped to the long arm of chromosome 4, at position 4q22.1, and Bin2 was expressed primarily in hematopoietic cells. The 4q22.1 region has been reported to be frequently disrupted in hepatocarcinoma and breast cancer (Rashid *et al.,* 1999; Schwendel *et al.,* 1998; Tirkkonen *et al.,* 1997; Yeh *et al.,* 1996). However, while we have obtained some results consistent with aberrant organization of the Bin2 gene in hepatoma cell lines, polymorphism cannot be ruled out, and we did not see any effect of ectopic Bin2 on the growth of hepatoma cells. Therefore, whether Bin2 losses or inactivation are relevant to malignancy is unclear. In contrast, a functional role for Bin2 in hematopoietic cells was supported by the finding that Bin2 expression is upregulated during granulocytic differentiation. In future work, it will be important to examine expression further by *in situ* methods to determine the range of potential Bin2 functions.

We found that Bin2 formed a stable biochemical complex with Bin1 but not with amphiphysin. Similar complexes have been reported between the BAR proteins Rvs161 and Rvs167 in yeast and amphiphysin and Bin1 in mammalian cells (Navarro *et al.,* 1997; Wigge *et al.,* 1997). The finding that amphiphysin did not interact with Bin2 is notable for several reasons. First, it supports the notion that Bin2 may not function in endocytosis. Second, it corroborates other evidence that BAR proteins including amphiphysin and Bin1 have unique functions, despite their structural similarities, insofar as Bin2 binds only Bin1. Third, this finding is consistent with observations in yeast that argue that BAR domains have overlapping but distinct func-

tions (Sivadon *et al.,* 1997). Finally, it suggests that BAR domains have specific oligomerization potentials, therefore raising the possibility that mammalian BAR protein functions are varied by combinatorial interactions. Given that amphiphysin and Bin2 are essentially tissue-restricted in expression, and possibly nonoverlapping, it is tempting to speculate that the function of Bin1 is differentially affected in tissues by coexpression with amphiphysin or Bin2.

We observed that Bin2–Bin1 association required N-terminal sequences within the BAR domain that include one of its two most highly conserved segments (aa 23–45). This segment is predicted to assume helical configuration, so it may participate in mediating association. In contrast, a second strongly conserved region of the BAR domain which is located in the C-terminal part (centered on aa 143–148) was dispensable for interaction, implying that it has a function other than mediating BAR protein oligomerization. Consistent with this likelihood, the C-terminal part of BAR in Bin1 is crucial for its tumor suppressor properties (Elliott *et al.,* 1999). While Bin2 is identical to Bin1 within this segment, it did not exhibit antiproliferative properties like Bin1. This difference in activity might reflect structural differences with Bin1 outside of the BAR domain, a possibility that could also be addressed by domain-swapping experiments. In future work, it will be important to define the physiological function of Bin2 by gene deletion in mice and to learn how Bin1 function is influenced by Bin2 interaction.

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Human Bin3 complements the F-actin localization defects caused by loss of Hob3p, the fission yeast homolog of Rvs161p

Eric L. Routhier[1], Timothy C. Burn[2], Ilgar Abbaszade[2],

Matthew Summers[3], Charles F. Albright[1] and George C. Prendergast[1,3]

[1]Cancer Research Group, Glenolden Laboratory, DuPont Pharmaceuticals Company, Glenolden PA 19036; [2]Applied Biotechnology Group, DuPont Pharmaceuticals Company, Wilmington DE 19803; and [3]The Wistar Institute, Philadelphia PA 19104

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*Corresponding author

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Phone: 610.237.7847

Fax: 610.237.7937

email: george.c.prendergast@dupontpharma.com

Summary

The BAR adaptor proteins Rvs167p and Rvs161p from *Saccharomyces cerevisiae* form a complex that regulates actin, endocytosis, and viability following starvation or osmotic stress. In this study, we identified a mammalian homolog of Rvs161p, termed Bin3 (Bridging INtegrator-3), and a *Schizosaccharomyces pombe* homolog of Rvs161p, termed Hob3p (Homolog Of Bin3). In mouse tissues, the Bin3 gene was expressed ubiquitously except for brain. *S. pombe* cells lacking Hob3p were often multinucleate and characterized by increased amounts of Calcofluor-stained material and mislocalized F-actin. For example, while wild-type cells localized F-actin to cell ends during interphase, *hob3*Δ mutants had F-actin patches distributed randomly around the cell. In addition, medial F-actin rings were rarely found in *hob3*Δ mutants. Notably, in contrast to *S. cerevisiae rvs161*Δ mutants, *hob3*Δ mutants showed no measurable defects in endocytosis or response to osmotic stress, yet *hob3+* complemented the osmosensitivity of a *rvs161*Δ mutant. Bin3 failed to rescue the osmosensitivity of *rvs161*Δ, but the actin localization defects of *hob3*Δ mutants were completely rescued by Bin3 and partially rescued by RVS161. These findings suggest that Hob3p and Bin3 regulate F-actin localization, like Rvs161p, but that other roles for this gene have diverged somewhat during evolution.

Introduction

BAR (Bin/Amphiphysin/Rvs domain) adaptor proteins, which include proteins encoded by the mammalian genes Amphiphysin, BIN1, and BIN2 and the *Saccharomyces cerevisiae* genes RVS167 and RVS161, are characterized by a unique N-terminal region termed the BAR domain. While their exact functions are largely unknown, BAR adaptor proteins appear to integrate signal transduction pathways that regulate membrane dynamics, F-actin cytoskeleton, and nuclear processes, roles that are highlighted in the nomenclature of two recently identified members of the family (Bridging INtegrators or BIN proteins).

Both genes encoding BAR adaptor proteins in *S. cerevisiae* were initially identified in a genetic screen for mutants that lost viability upon nutrient starvation [1; 2]. Subsequent work revealed that Rvs167p and Rvs161p form a physiological complex that regulates F-actin localization, cell polarity, bud formation, and endocytosis [2-7]. Rvs161p is also important for karyogamy, the nuclear fusion process which follows mating [8]. A variety of Rvs-interacting proteins were identified that are consistent with Rvs161p and Rvs167p functions in F-actin regulation, lipid metabolism, cell cycle integration, and nuclear processes [9-13]. Despite the importance of Rvs161p and Rvs167p in these diverse functions, RVS161 and RVS167 genes are not required for viability.

Three genes encoding BAR adaptor proteins have been described in mammalian cells.

Two of these genes, amphiphysin and BIN1, encode structural orthologs of Rvs167p, whereas the third gene, BIN2, encodes a structurally unique protein. The expression patterns of each gene suggest different physiological roles: BIN1 is widely expressed whereas amphiphysin and BIN2 are tissue-restricted in their expression. The product of the amphiphysin gene, which was identified by virtue of its biochemical properties [14], is a neuronal adaptor protein that regulates synaptic vesicle endocytosis [15]. The restricted pattern of amphiphysin expression argues that its physiological function is limited to the specialized processes of synaptic vesicle recovery. In a similar way, BIN2 expression is restricted to hematopoietic cells. BIN2 function is undefined but appears to be nonredundant with other mammalian BAR proteins [16]. The BIN1 gene has a complex function(s) suggested by its diverse patterns of alternate splicing. BIN1 splice isoforms have been identified by virtue of interaction with the c-Myc oncoprotein, structural similarity to amphiphysin, interaction with the nuclear tyrosine kinase c-Abl, and characterization of the BIN1 gene itself [17-23]. Brain-specific isoforms, alternately termed amphiphysin II or amphiphysin-like isoforms, are exclusively cytosolic and can influence endocytosis [15]. However, only brain isoforms include regions required for interaction with key components of the endocytosis machinery [24]. Thus, it is unclear whether Bin1 participates in endocytosis outside the brain. Nuclear functions are suggested by the ability of muscle-specific and ubiquitous isoforms to localize to the nucleus and to functionally associate with the c-Myc and c-Abl proteins [17; 19; 25]. In particular, c-Myc-interacting isoforms have tumor suppressor and transcriptional properties that impact cell differentiation and cell death decisions [17; 25-31].

To further investigate the function of BAR adaptor proteins, we identified a mammalian homolog of Rvs161p, termed Bin3 (Bridging INtegrator-3), and a *Schizosaccharomyces pombe* homolog of Rvs161p, termed Hob3p (Homolog Of Bin3). Analysis of *hob3*Δ mutants revealed an important role for Hob3p in regulation of F-actin localization, as was found for Rvs161p. The F-actin localization defect of *hob3*Δ mutants was completely rescued by human BIN3 and partially rescued by RVS161, raising the possibility that Bin3 regulates F-actin localization in mammalian cells.

Materials and Methods

Cloning. The *S. pombe hob3+* gene was identified through BLAST [32] searches of the *S. pombe* genome using the *S. cerevisiae* RVS161 gene as query. The *hob3+* gene was cloned by PCR from a stationary-phase, *S. pombe* single-stranded cDNA library (Library-In-A-Tube", QBiogen), using oligonucleotide primers derived from the *hob3+* locus. Sequences encoding the human Bin3 protein were similarly identified by TBLASTN [32] searches of the translated EST database using the *S. cerevisiae* Rvs161p as a query. Human BIN3 cDNA was cloned by PCR from single-stranded Library-In-A-Tube" cDNA libraries (QBiogen). Information obtained from the EST databases was used to construct a full-length cDNA clone. Sequence determinations included the full-length IMAGE EST clones obtained from Research Genetics (Huntsville AL). Human BIN3 cDNA was subcloned as an untagged or hemagglutinin (HA)-tagged insert into pcDNA3/neo (Invitrogen) and these plasmids were used for PCR to amplify BIN3 cDNA for insertion into yeast vectors. cDNAs were digested with NdeI and PspAI or BamHI and cloned into the *S. pombe* expression plasmid pREP2 [33]. Gene deletions in *S. pombe* were performed as described [34] using plasmid pFA6a-kanMX6-HA as a template for the construction of a disrupted allele. BAR protein-encoding cDNAs were cloned between the XbaI and BamHI or SmaI sites of the 2μ-based budding yeast expression vector YEp195-ACN (courtesy J. Toyn). Yeast transformations were performed by standard methods [35; 36]. Oligonucleotide sequences are available upon request.

Strains and media. *S. pombe* strains FY71 (*h-, ade6-M216, leu1-32, ura4-D18)* and FY72 (*h+, ade6-M210, leu1-32, ura4-D18*) were obtained courtesy of S. Henry, Mellon College. Strain ELR6 (*ade6-M210, leu1-32, ura4-D18, hob3Δ::kanMX6*) is a derivative of diploid strain KGY246/249 [37], obtained by one-step gene disruption. Integration of the altered allele by homologous recombination was verified by Southern blotting using a ^{32}P-labelled PCR product derived from the 5' untranslated region of the *hob3+* locus, extending from 280 bp to 986 bp from the putative start codon of *hob3+* [38]. The probe was labelled with the High Prime DNA Labelling Kit (Roche Molecular Biochemicals) and α-^{32}P-dCTP (NEN). *S. cerevisiae* strains BY4741 (MAT*a, ura3, leu2, his3, met15*) and BY4741-3489 *(*MAT*a, ura3, leu2, his3, met15, rvs161Δ::kanR*) were obtained from J. Toyn, Applied Biotechnology Group, DuPont Pharmaceuticals Company. *S. pombe* strains were grown in YE medium or EMM2 containing appropriate nutritional supplements when necessary [39]. Expression from pREP2 plasmids was achieved by growing cells to early log phase in medium containing 0.06 mM thiamine, washing the cells 3x in thiamine-free medium, and resuspending the cells in the same medium. Budding yeast were grown in YPAD or SC medium lacking the appropriate nutritional supplements, in some cases with the addition of 6% (w/v) NaCl [40].

Immunofluorescence. Exponential phase *S. pombe* cultures were stained for F-actin as described [41] using AlexaFluor 488-conjugated phalloidin (Molecular Probes). Nuclei were stained with DAPI. Images were captured on a Nikon Eclipse TE300 microscope fitted with a Nikon Plan Fluor 100X objective using a Toshiba 3CCD camera. Images were manipulated using Image Pro Plus version 4.0 software (Media Cybernetics).

Endocytosis. Exponential phase cultures of *S. pombe* cells were assayed for uptake of the lipophilic styryl dye FM4-64 (Molecular Probes) as described [42].

Northern Analysis. MTNI and MTNII human multiple tissue Northern blots obtained from Clontech (Palo Alto CA) were hybridized to ^{32}P-labelled probes for Bin3, Bin1, and amphiphysin I generated by the random priming method as per the vendor's instructions. The Bin1 and amphiphysin I probes have been described [14; 27]. The Bin3 probe was a ^{32}P-labelled 600 bp BamHI-BglII fragment of the human BIN3 cDNA. Hybridization of a Northern blot of RNA isolated from a panel of tumor cell lines, cultured and processed as described previously [17; 31], was performed using the BIN3 probe and a β-tubulin probe to normalize the blot.

Results

Bin3 encodes a widely expressed BAR adaptor protein related to Rvs161p. Sequences encoding Bin3, a novel human BAR adaptor protein, were identified using Rvs161p to search the EST database with the TBLASTN algorithm. Sequence analysis of full-length cDNA clones identified in this manner revealed that Bin3 was a protein of 253 residues in length and was comprised solely of a BAR domain, like Rvs161p (Fig. 1a, 1b). The Bin3 BAR domain was 27% identical to Rvs161p but less than 24% identical to other BAR domains (Table 1). Northern analysis of mouse tissue RNAs was performed to compare the Bin3 expression pattern to that of other mammalian BAR adaptor genes. A single mRNA species of ~2.2 kb was detected at similar levels in all embryonic and adult tissues examined, except for brain where Bin3 mRNA was undetectable (Fig. 2a). This wide expression pattern of Bin3 was similar to Bin1, which was widely expressed, but contrasted with amphiphysin, which was expressed primarily in brain, and Bin2, which was expressed primarily in hematopoetic cells. Since Bin1 expression was frequently decreased in malignant cells, Bin3 expression was determined in a panel of human tumor cell lines. All cell lines tested expressed Bin3 (Fig. 2b). We concluded that Bin3 was a widely expressed, BAR adaptor protein that was structurally most similar to *S. cerevisiae* Rvs161p.

***S. pombe hob3+* encodes a BAR adaptor protein related to Rvs161p and Bin3.** Sequences encoding Hob3p (Homolog of Bin3) were identified using Rvs161p to search the *S.*

pombe genome (Fig. 1a). Hob3p was 264 residues in length, 56% identical to *S. cerevisiae* Rvs161p, and 29% identical to Bin3 throughout its entire sequence (Fig. 1b). In contrast, the Hob3p BAR domain sequences were less than 26% identical to the BAR domain sequences in Bin1, Bin2, and amphiphysin (Table 1). Like Rvs161p and Bin3, Hob3p was comprised solely of a BAR domain, without the additional C-terminal sequences found in Rvs167p or known mammalian BAR adaptor proteins (Fig. 1b). The similarity of BAR sequences and lack of non-BAR sequences suggest that Rvs161p, Bin3, and Hob3 comprise a subfamily within the family of BAR adaptor proteins. The other BAR adaptor protein encoded by the *S. pombe* genome was identifed. The structure and characterization of this predicted protein, which was most similar to Rvs167p, will be described elsewhere[2].

***hob3*Δ mutants have a cell division defect.** We began by studying *S. pombe hob3+* since fission yeast genetics allowed us to rapidly characterize Hob3p function. Using standard methods, haploid *S. pombe* strains were made where the entire coding region of *hob3+* was replaced with the *kanMX6* cassette, conferring resistance to G418 [34]. Southern analysis confirmed construction of a strain with the *hob3*Δ allele (Fig. 3a). Examination of *hob3*Δ mutants revealed a fraction of cells that were longer than *hob3+* cells and contained more than two nuclei (Fig. 3b). In particular, about 9% of *hob3*Δ cells from an actively-growing culture contained more than two nuclei, with most of these elongated cells containing four nuclei (n = 108). In contrast, no cells with more than two nuclei were observed in a parallel culture of *hob3+* cells (n = 125). Calcofluor staining showed that septal material separated most of the nuclei in *hob3*Δ cells (Fig. 3b). Furthermore, the *hob3*Δ cells contained increased amounts of Calcofluor-

41% had medial F-actin patches in both compartments with an F-actin ring, 32% had delocalized F-actin patches in both compartments, and 27% had medial F-actin patches in one compartment with delocalized F-actin patches in the other compartment (n = 22). These findings contrast with *hob3+* cells where 100% of cells containing two nuclei exhibited medial F-actin staining (n = 29). Hence, Hob3p plays an important role in the localization of F-actin in interphase and mitotic cells. Consistent with loss of cell polarity and consequent abnormally shaped cells observed in mutants of the *S. pombe* F-actin-encoding *act1+* gene [47], we observed such misshapen cells in *hob3Δ* cultures (Fig. 4). Specifically, 13% of cells (n = 116) in Fig. 4 lost their cylindrical appearance and took on a rounded appearance, versus 0% of cells in a matched *hob3+* culture (n = 163). Loss of shape was not limited to mononuclear cells; 48% of misshapen cells possessed two or more nuclei. F-actin delocalization was observed in all misshapen cells. We concluded that *hob3+* was necessary for F-actin regulation and completion of septation, the process of cytokinesis in fission yeast cells.

***hob3Δ* mutantsrespondnormallytonutrientandosmoticstress.** RVS161 was discovered in a screen for mutants that had reduced viability upon starvation for glucose, nitrogen or sulfur [1]. In these experiments, *rvs161Δ* mutants had a 35% reduction in cell viability after 48 hours in N005 low nitrogen medium. Further analysis revealed that *rvs161Δ* mutants showed dramatic morphologic changes in response to high salt and low nitrogen media, and more significant reductions in cell growth when shifted to media with high salt [48]. Based on these results, the response of *hob3Δ* mutants to nutrient and osmotic stress was tested. This analysis revealed that *hob3Δ* mutant cells were relatively insensitive to lack of nitrogen or elevated/decreased

temperature, as assayed by growth on plates (Fig. 5). Furthermore, microscopic inspection of *hob3*Δ mutant cells following temperature, osmotic, or nutrient shift did not reveal detectable differences in cell morphology (data not shown). Based on these results, we concluded that *hob3*Δ mutants, unlike *rvs161*Δ mutants, respond like *hob3*+ cells to changes in temperature, osmolarity, and nutrients.

***hob3*Δ mutants undergo normal fluid-phase endocytosis.** *rvs161*Δ mutants were defective in fluid-phase and receptor-mediated endocytosis [2; 8]. To measure the rate of fluid-phase endocytosis in *hob3*Δ mutants, the ability of cells to accumulate FM4-64 was quantified. FM4-64 is a fluorescent lipophilic styryl dye which specifically accumulates in vacuolar membranes of both budding and fission yeasts [42; 49]. When added to cells, FM4-64 initially stained the plasma membrane (Fig. 6). Within 15 minutes, FM4-64 internalized at the cell ends in presumed endocytic vesicles (Fig. 6). During the next 60 minutes, the number of FM4-64 staining structures decreased to 2-3 per cell and their size increased. Based on published data, these final structures were vacuoles. A comparison of *hob3*+ and *hob3*Δ mutants at several times did not reveal any detectable differences in the kinetics or morphology of the FM4-64 staining structures. Based on these results, we conclude that fluid-phase endocytosis was normal in *hob3*Δ mutants.

F-actin localization defects in *hob3*Δ mutants are completely rescued by Bin3 and partially rescued by RVS161. The structural similarities between Hob3p, Bin3, and Rvs161p as well as the functional similarities between *rvs161*Δ mutants and *hob3*Δ mutants, suggested that

Hob3p, Bin3, and Rvs161p share common functions. To test this hypothesis, we determined whether ectopic expression of Bin3 and Rvs161p could rescue the defects of *hob3*Δ mutants. For this purpose, plasmids were constructed where Hob3p, Bin3, Rvs161p, and Rvs167p were expressed using the thiamine-repressible *nmt1* promoter of *S. pombe* [33]. These plasmids or a control plasmid were then introduced into *hob3*Δ mutants and the fraction of elongated cells and F-actin staining patterns were quantified (Table 2, Fig. 7). As expected, ectopic expression of *hob3+* complemented the cell elongation and F-actin defects in *hob3*Δ cells whereas the control vector had no effect. Interestingly, Bin3 expression also corrected the cell elongation and F-actin defects of *hob3*Δ mutants while Rvs161 expression partially corrected the defects of *hob3*Δ mutants. In particular, *hob3*Δ mutant cells expressing Rvs161p were not elongated and contained easily detectable medial F-actin in mitotic cells. Rvs161p expression failed, however, to correct the mislocalization of F-actin patches in *hob3*Δ mutants. Rescue of *hob3*Δ mutants by Bin3 and Rvs161p was specific for these BAR adaptor proteins since Rvs167p expression did not correct the defects of *hob3*Δ mutants. We conclude that Bin3 and Rvs161p, but not Rvs167p, at least partially rescue the F-actin localization defects of *hob3*Δ mutants arguing that these proteins can perform similar functions.

***hob3+*, but not Bin3, complements the osmotic sensitivity of *S. cerevisiae rvs161*Δ mutants.** Since both RVS161 and BIN3 complemented the F-actin localization defects of *hob3*Δ mutants, we tested whether expression of BAR-containing proteins could rescue a *S. cerevisiae rvs161*Δ mutant. Complementation was tested by the ability of a *rvs161*Δ strain to grow on synthetic dropout medium containing 6% NaCl. As expected, *rvs161*Δ cells lacking a plasmid or

containing a control plasmid failed to grow on media with 6% NaCl (Fig. 8). In contrast, *rvs161*Δ mutants expressing Rvs161p or Hob3p, but not Bin3 or Bin1, grew similarly to RVS161 cells (Fig 8). All strains which received a plasmid grew on synthetic dropout medium lacking 6% NaCl (data not shown). We conclude that, due to the lesser divergence between the Rvs161p and Hob3p proteins, Hob3p was able to complement the osmolarity defect of *rvs161*Δ null cells, but that the greater extent to which Rvs161p and Bin3 have diverged precluded complementation by Bin3.

Discussion

While the exact role of the N-terminal fold of the BAR family proteins is unknown, it is apparent that BAR family proteins are nonredundant in function. However, the BAR family may be subdivided based on structural considerations. Thus, a subset of BAR family members contain a C-terminal SH3 domain. In some cases, as for the Bin1-binding c-Abl oncoprotein, the protein partner responsible for interaction is known [19]. Another subset of BAR family members contain domains known to be involved in binding components of the endocytotic machinery and vesiculation [14; 22; 50]. Other domains, such as the c-Myc binding domain of Bin1 [17], are unique within the BAR family. The proteins described in the present study are characterized by a lack of identifiable functional domains outside of the BAR N-terminal fold. In combination with the greater homology exhibited between members of this subset and their ability to cross-complement, this suggests to us that they form a bona fide subfamily within the BAR family of proteins. The inability of other BAR-containing proteins to complement defects

in the expression of these proteins, even in the case of proteins native to the same organism, supports this notion[2] [48]. While it is known that some members of the BAR family are able to interact, such as the yeast Rvs161p and Rvs167p proteins [7; 51], and amphiphysin and the brain isoform of Bin1 [22], the possibility of homo- or heterotypic interactions between other BAR family members remains to be determined.

As the case with Rvs161 in budding yeast, the phenotype caused by *hob3+* deletion in fission yeast was linked to cytoskeletal actin regulation. The presence of multiple Calcofluor-reactive primary septa in *hob3Δ* mutant cells coupled with the observation of an actin localization defect is reminiscent of the phenotypes exhibited by known cell separation and actin regulatory mutants of *S. pombe*. Thus, inactivating mutations of the *sep2+, sep12+* , *spn1+ and rlc1+* genes of *S. pombe* result in linear, multiseptated cells, in most cases with increased deposition of septal material [52-55]. *sep2+* was identified in a screen for mutants with increased resistance to lysing enzymes, while *sep12+* was isolated by application of the diploid enrichement screen of Chang *et al* [56]. It is worth noting that a fraction of *sep2* cells contained double septa, which yielded two daughter cells and an anucleate minicell upon cleavage. Neither double septa nor anucleate cells were observed in cultures of *hob3Δ*. It was shown that cultures of *sep12* mutant cells contained 64% hyphae, a much larger percentage than the typical 10-15% observed in *hob3Δ* cultures. In addition, *sep12* cells were sterile, while *hob3Δ* mated with normal kinetics. *spn1+* is a member of the *S. pombe* septin family of proteins. As such, it has a role in promoting septation of fission yeast. However, a role in actin patch movement has not been predicted. *rlc1+* encodes a myosin regulatory light chain which associates with the yeast

Myo2p and Myo3p gene products. Although the morphology of *hob3*Δ cells closely resembled that of *rlc1* mutant cells, the latter were found to be cold-sensitive for growth, a condition not seen in *hob3*Δ cultures. Interestingly, Rvs161p, Hob3p and Bin3 are homologous to unconventional myosins; Rvs161p is 25% identical to Myo1p, the sole type II unconventional myosin of budding yeast; Hob3p is 20% identical to Myo2p, one of two myosins in *S. pombe*, and Bin3 is 24% identical to human type VI unconventional myosin. BLAST analysis of the Bin3 protein assigns unconventional type VI myosins as the most highly homologous non-BAR polypeptides.

The mislocalization of F-actin patches by *hob3*Δ null cells has been previously observed in mutants of the Arp2/3 complex of *S. pombe*, as well as in mutants of other, known actin-interacting proteins such as the products of the *cdc3+* and *cdc8+* genes, which encode profilin and tropomyosin, respectively [44; 45; 57; 58]. However, these mutants do not display the linear, multiseptated morphology characteristic of *hob3*Δ cells. In addition, loss of Cdc3p, Cdc8p or Arp3p function is lethal, whereas loss of Hob3p is not. Given that the major defect in these mutants is probably actin-related, and that perturbations of actin organization generally result in gross morphological defects throughout the cell cycle, it is not surprising that the *hob3+* gene is not essential, nor do *hob3*Δ cells exhibit the profound morphological abnormalities observed in more severe cases of loss of actin organization, such as in the *cdc3* mutant [58]. Nevertheless, a defect in F-actin patch movement is apparent in *hob3*Δ cells. It remains to be determined whether Hob3p is directly associated with actin or with actin-binding proteins such as profilin, tropomyosin or the Arp2/3 complex.

We observed cross-species complementation of the *hob3*Δ F-actin defect by the budding yeast homolog Rvs161p , and by the mammalian homolog Bin3. A partial rescue of the F-actin localization defect by Rvs161p was observed insofar as the majority of dividing cells regained medial F-actin staining, but failed to correctly localize F-actin patches during interphase. On the other hand, Bin3 was able to rescue both loss of medial F-actin and localization of F-actin to cortical patches during interphase. It was noted that budding yeast Rvs167p, which is not a member of the subfamily of BAR proteins defined by Rvs161p, Hob3p and Bin3, was unable to rescue the F-actin defect of *hob3*Δ cells. An alternative explanation for the partial complementation observed with Rvs161p and the lack of complementation seen in the case of Rvs167p could be due to decreased steady-state levels of these proteins in *S. pombe*. However, we have confirmed the presence of either Bin1 or Bin3 polypeptides in *hob3*+ and *hob3*Δ *S. pombe* strains transformed with pREP2-based expression vectors. Furthermore, we were able to ascertain that the resulting Bin1 polypeptide failed to correct the F-actin defect observed in *hob3*Δ cells (data not shown). We thus favor the interpretation that Bin3 is the mammalian homolog of Rvs161p and Hob3p, but that there exists a degree of divergence in this gene during evolution. For example, Bin3 and Hob3p share important roles with Rvs161p in the control of the actin cytoskeleton, but only Rvs161p exhibits a role in endocytosis, and only Hob3p exhibits a role in cell division. Despite the lack of any role in cell division in budding yeast, RVS161 complemented the defects in this process caused by *hob3*Δ gene deletion as well as Bin3. In support of the notion of some evolutionary drift in the function of this gene during evolution, Bin3 was found to exhibit a unique localization in human cells to mitochondria and Golgi rather than to sites of actin polymerization as in the case of Rvs161p and Hob3p in budding and fission

yeasts[3]. Thus, a major finding of our study is that while Bin3 is clearly homologous to yeast Rvs161p and Hob3p at some levels, it is also clear that the function of this BAR adaptor protein has diverged to some extent and/or is being utilized differently in cells during evolution. Further insights into the exact mechanistic role of Bin3 in cell division processes will require studies in mouse cells in which the Bin3 gene has been targeted for homozygous deletion.

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Footnotes

[1]Abbreviations used are: EST, expressed sequence tag; PCR, polymerase chain reaction; YE, yeast extract medium; EMM2, Edinburgh minimal medium; EMM2-N, Edinburgh minimal medium without NH_4Cl; EMMG, Edinburgh minimal medium without NH_4Cl, with glutamtate; YPAD, yeast complete medium with adenine; SC, synthetic complete yeast medium; DAPI, 4',6'-diamidino-2-phenylindole.

[2]E.L.R., C.F.A. and G.C.P., manuscript in preparation.

[3]J.B. DuHadaway and G.C.P., unpublished observations

[4]The nucleotide sequences reported in this paper have been deposited in the GenBank" database under GenBank Accession Number AF2717232 (human BIN3 cDNA), AA418871 (human BIN3 EST), AAF76218 (human Bin3 protein), AF271733 (murine Bin3 cDNA), AF275638 (*S. pombe hob3+* cDNA) and AAF86459 (*S. pombe* Hob3p). The sequence alignment in Fig. 1 included the budding yeast protein Rvs161p, available from the Swiss Protein Database as Swiss-Prot # 25343.

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Figure Legends

Figure 1. Bin3 and Hob3p are members of the BAR family. **(a)** Alignment of Bin3 with *S. pombe* Hob3p and *S. cerevisiae* Rvs161p. Identical residues in all sequences are contained in black boxes, while residues conserved between at least 50% of sequences are shown enclosed by gray boxes. **(b)** Cartoon depicting known BAR family proteins. The N-terminal BAR fold is shown in blue. Domains known to interact with c-Myc are in gray, while those implicated in endocytosis are in red. SH3 domains are depicted in yellow.

Figure 2. Distribution of mammalian BAR mRNAs in normal tissues and tumors. **(a)** Multiple tissue northern blots probed for Bin1 mRNA (top panel), Bin3 mRNA (middle panel), and amphiphysin mRNA (lower panel). **(b)** Bin3 mRNA expression in tumor cell lines (upper panel). The membrane was stripped and reprobed for β-tubulin mRNA as a loading control (lower panel). WM164 and WM1341D were derived from metastatic melanoma, while C33A, MS751, SiHa and HeLa were isolated from cervical carcinomas. A549 is a lung carcinoma line, while HepG2 originates from hepatocellular carcinoma. The C2C12 cell line is an undifferentiated mouse myoblast line.

Figure 3. Generation of a fission yeast strain harboring a deletion of the mammalian BIN3 homolog. **(a)** Southern blot of BamHI-digested genomic DNA from haploid *hob3+* (right lane) and *hob3Δ* (left lane). **(b)** Morphology of the *hob3Δ* strain. Exponentially growing cells were stained with Calcofluor and DAPI. **(c)** Growth kinetics and viability of *hob3Δ* null cells. Graph

of cell density and viability versus time for exponential phase cells inoculated into YE medium. Cell density was determined by counting appropriate dilutions of cells with the aid of a hemocytometer. Viability was determined by a colony formation assay on solid YE medium. All measurements were obtained in triplicate.

Figure 4. Deletion of *hob3+* results in F-actin localization and septation defects. Exponentially growing *S. pombe* cells were fixed and stained with AlexaFluor 488-phalloidin to visualize polymerized actin (top panels). DNA was stained with DAPI (bottom panels). *hob3+* cells are depicted in the left panels, while *hob3*Δ cells are shown in the right panels. Hazy shading observed in some areas of the figures are due to the presence of cells in a different focal plane with repect to the majority of cells. Note that this effect is more pronounced in the *hob3*Δ strain due to the propensity of these cells to grow in clumps. *Insets*, smaller fields were magnified to highlight differences in F-actin staining. Arrows point to medial F-actin patches and rings. Asterisks indicate mislocalized F-actin patches.

Figure 5. *hob3*Δ null cells do not display reduced viability upon starvation. Effect of temperature and lack of nitrogen (EMMG) on growth on solid medium. Exponentially growing cells of each strain from liquid YE cultures were counted in triplicate. $1X10^4$ to $1X10^1$ cells of each strain were inoculated onto the indicated medium in tenfold dilutions from left to right, and the plates were allowed to grow at the indicated temperature until colonies formed (3-12 days).

Figure 6. *hob3*Δ null cells lack gross endocytotic defects. FM4-64 uptake assay of

exponentially growing cells. Cells were incubated at room temperature in the presence of FM4-64 and photographed at the indicated times.

Figure 7. Ectopic expression of Bin3 and Rvs161p complements the F-actin defect of a *hob3*Δ null mutant. A *hob3*Δ null strain was transformed with pREP2-based vectors expressing BAR family proteins. Exponentially growing transformants were stained with AlexaFluor 488-phalloidin to visualize polymerized actin. pREP2 is the empty expression vector. pREP2-*hob3*+, pREP2-RVS161, pREP2-BIN3 and pREP2-RVS167 encode the *hob3*+, RVS161, BIN3 and RVSD167 cDNAs, respectively. Contractile rings are indicated by arrows. Note delocalized cortical actin patches aligned along the length of the cells denoted by asterisks.

Figure 8. The high salt intolerance of *rvs161*Δ can be complemented by ectopic expression of the fission yeast homolog *hob3*+. RVS161 strain BY4741 and *rvs161*Δ strain BY4741-3489 were transformed with the indicated plasmids. Transformants were streaked onto synthetic uracil dropout medium containing 6% NaCl and incubated at 30 C for 7 days. Designations following YEp195-ACN indicate the identity of the encoded BAR protein.

(a)

Fig. 1

```
Bin3          1 MSWIPFK.....IQQP...KKQIVPKTVERDFEREYGKLQQLEEQTRRLQKDMTKSTDADLAMSKSAVKTSLDLLSNPLC
Hob3p         1 MSWEQFKKAVNRACTSVMMKTCHVERTVDREFETEERRYRTMESAAKKLQKEAKGYLDALRAMTASQTRIA.NTIDAFYG
Rvs161p       1 MSWEQFKKAINRAGHRVIIK..NVDKTIDKEYDMEERRYKVLQRAGEALQKEAKGFLDSLRAVTASQTTIA.EVISNLYD

Bin3         73 EQDQDLLNMVTALDTAMKRMDADFNQEKVMQI....QKTVIEFLKKFGSVFTSLNMAVKRREQALQDYRRLQAKVEKYEEK
Hob3p        80 D....AKSKDGVSAVYRQVVEDLDADTVKELDGPFRTTVLDPISRFGSVFPDINAAITKRNHKLLDHDAMRAKVQKLVDK
Rvs161p      78 DSKYVAGGGYNVGNYYLQGVQDFDSETVKQLDGPLRETVLDPITKFSTYFKEIEEAIKKRDHKKQDFDAAKAKVRRLVDK

Bin3        149 EKTGFVLAKLHQAREELRPVREDFEAKNRQLLEEMPRFYGSRLDYFQPSFESLIRAQVVYYYSENHKIFGDLSHQLDQFGE
Hob3p       156 FSNE..TTKLFRTEKEAAMAKEVYETLNNQLVSELPQLIALRVPYLDPSSFEALVKIQLRFGREGYEKMAQVGQYFDNSVR
Rvs161p     158 DAKD..ASKLIRAEKELSLAKDIFENLNNQLKTELTQLVSLRVTYFDTSFEALIKIQLRFGTDGYTRLAQIQQYLDQQSR

Bin3        239 SDEQR...ERENEAKLSELRALSIVA...DD
Hob3p       234 EDYSNGLLLDKVEQVLQSMRDLSIAGLNNSQ
Rvs161p     236 DDYANGLLLDTKIEELLGQMTSLDICAL.GIK
```

(b)

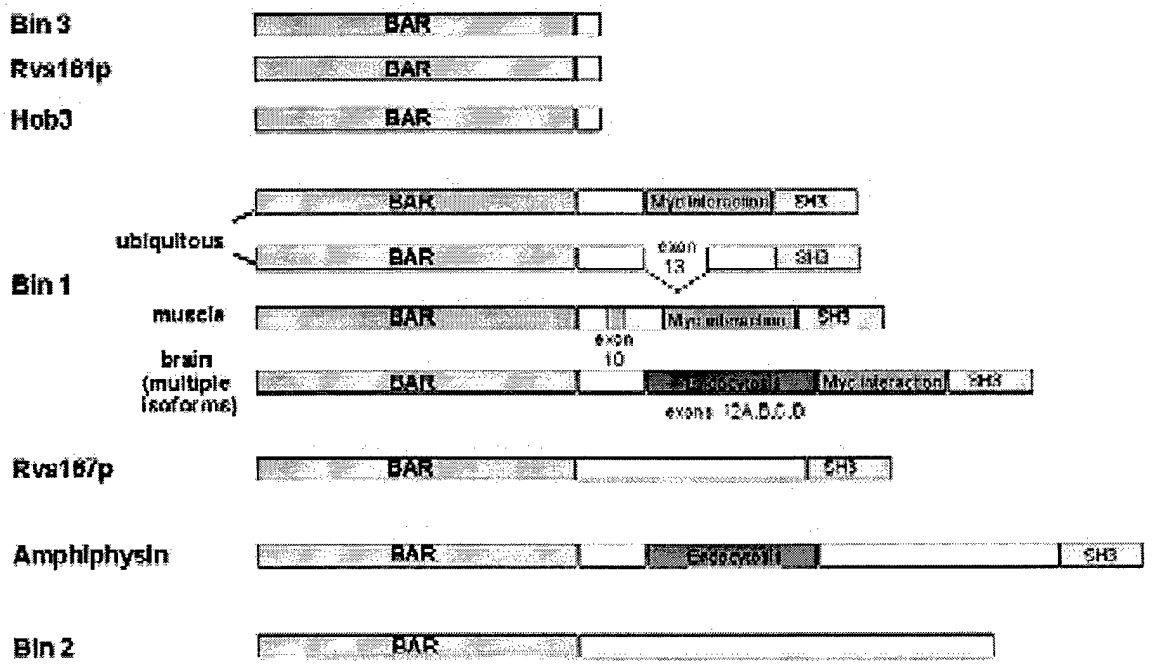


Fig. 2

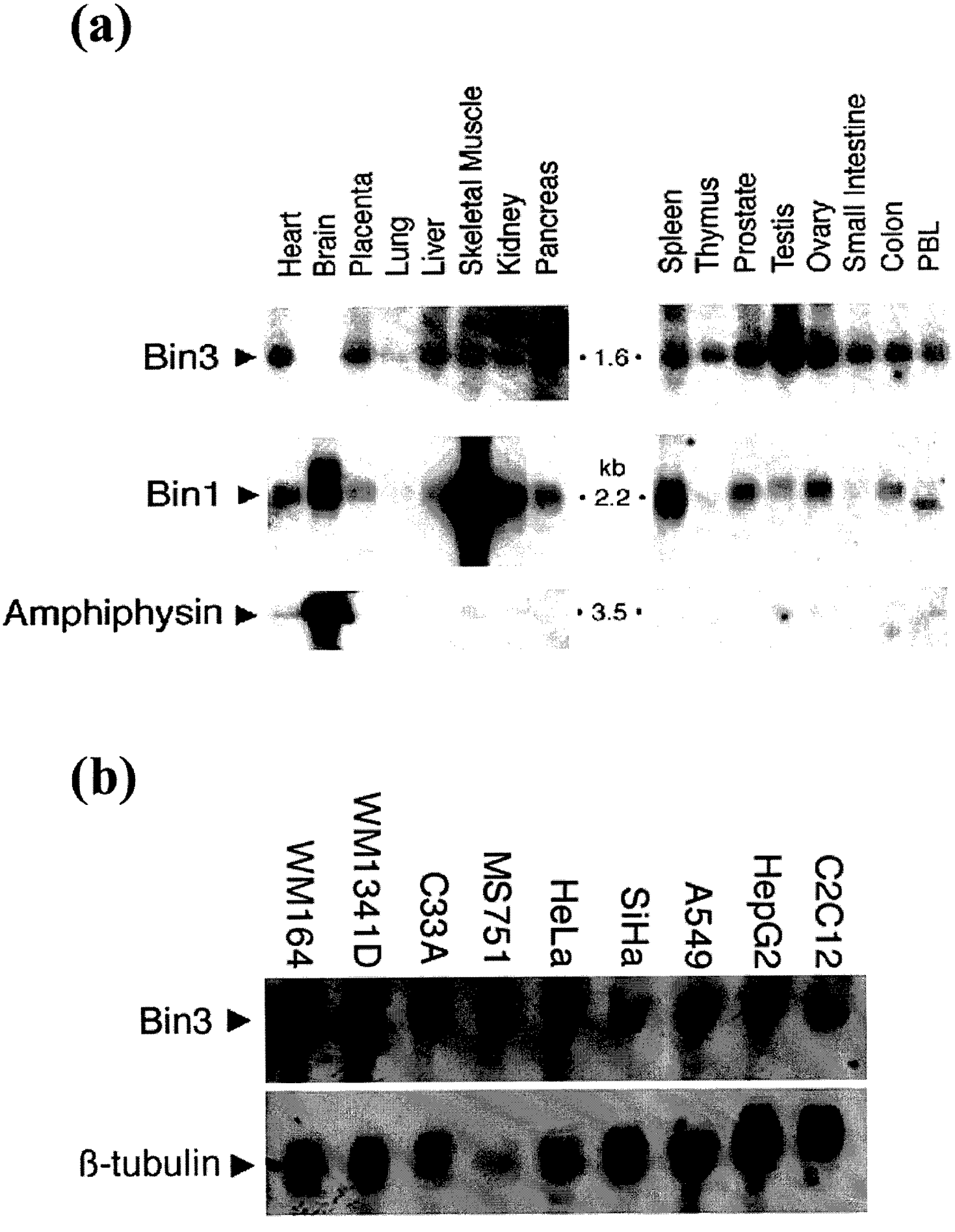
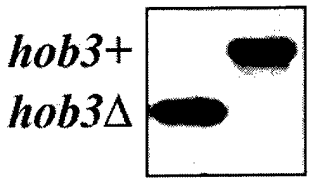
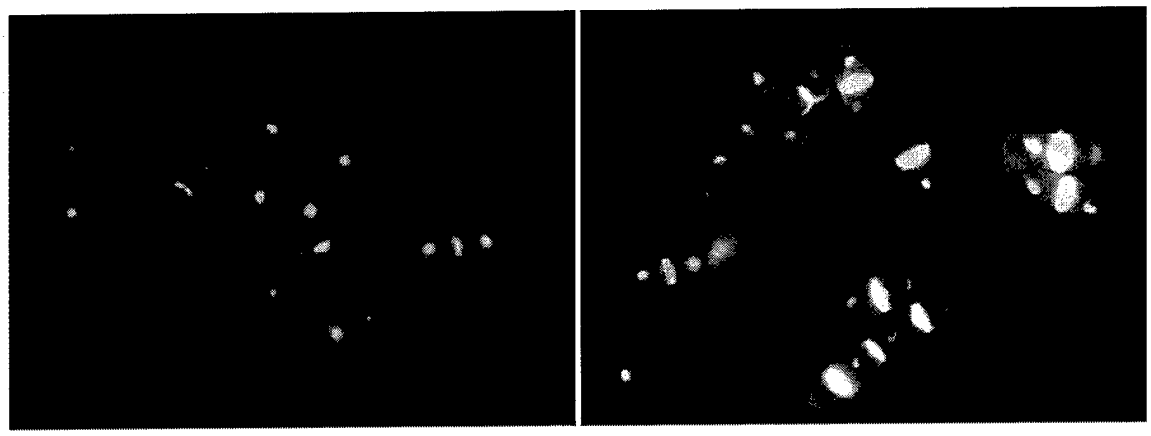

(a)
Heart
Brain
Placenta
Lung
Liver
Skeletal Muscle
Kidney
Pancreas
Spleen
Thymus
Prostate
Testis
Ovary
Small Intestine
Colon
PBL
Bin3
1.6
kb
Bin1
2.2
Amphiphysin
3.5
(b)
WM164
WM1341D
C33A
MS751
HeLa
SiHa
A549
HepG2
C2C12
Bin3
ß-tubulin

Fig. 3

(a)


hob3+
hob3Δ

(b)


hob3+
hob3Δ

(c)

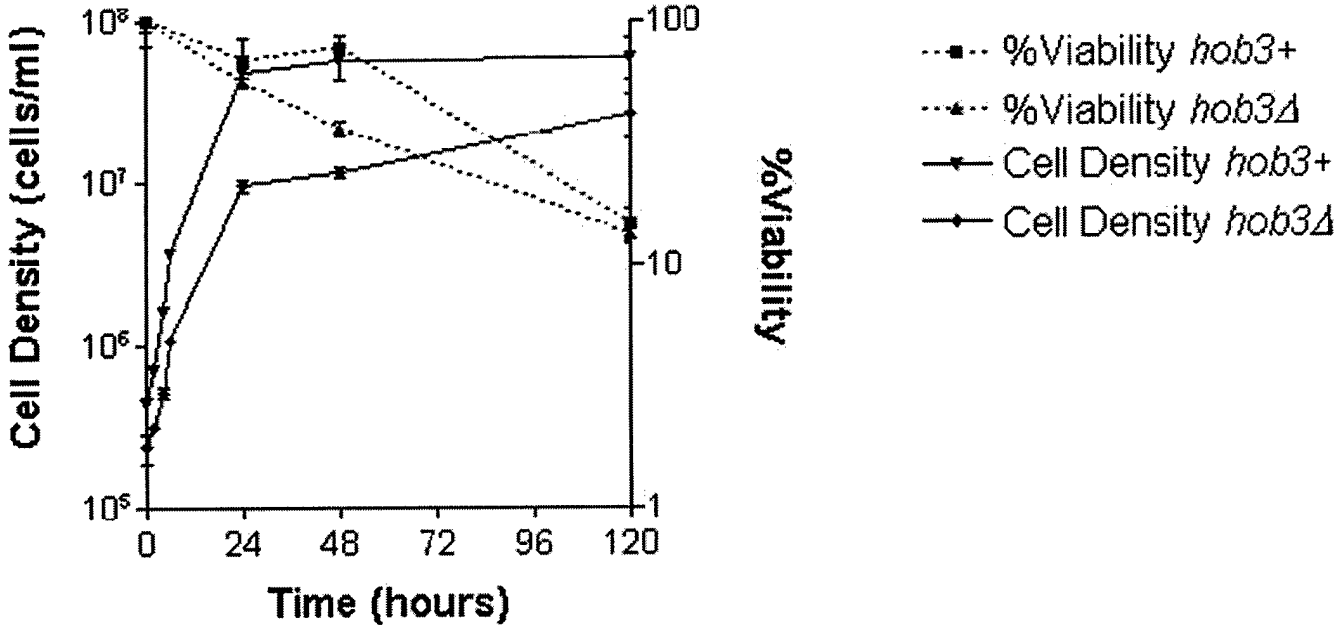

Cell Density (cells/ml)
10⁸
10⁷
10⁶
10⁵
100
10
1
%Viability
0
24
48
72
96
120
Time (hours)
%Viability hob3+
%Viability hob3Δ
Cell Density hob3+
Cell Density hob3Δ

Fig. 4

hob3+

hob3Δ

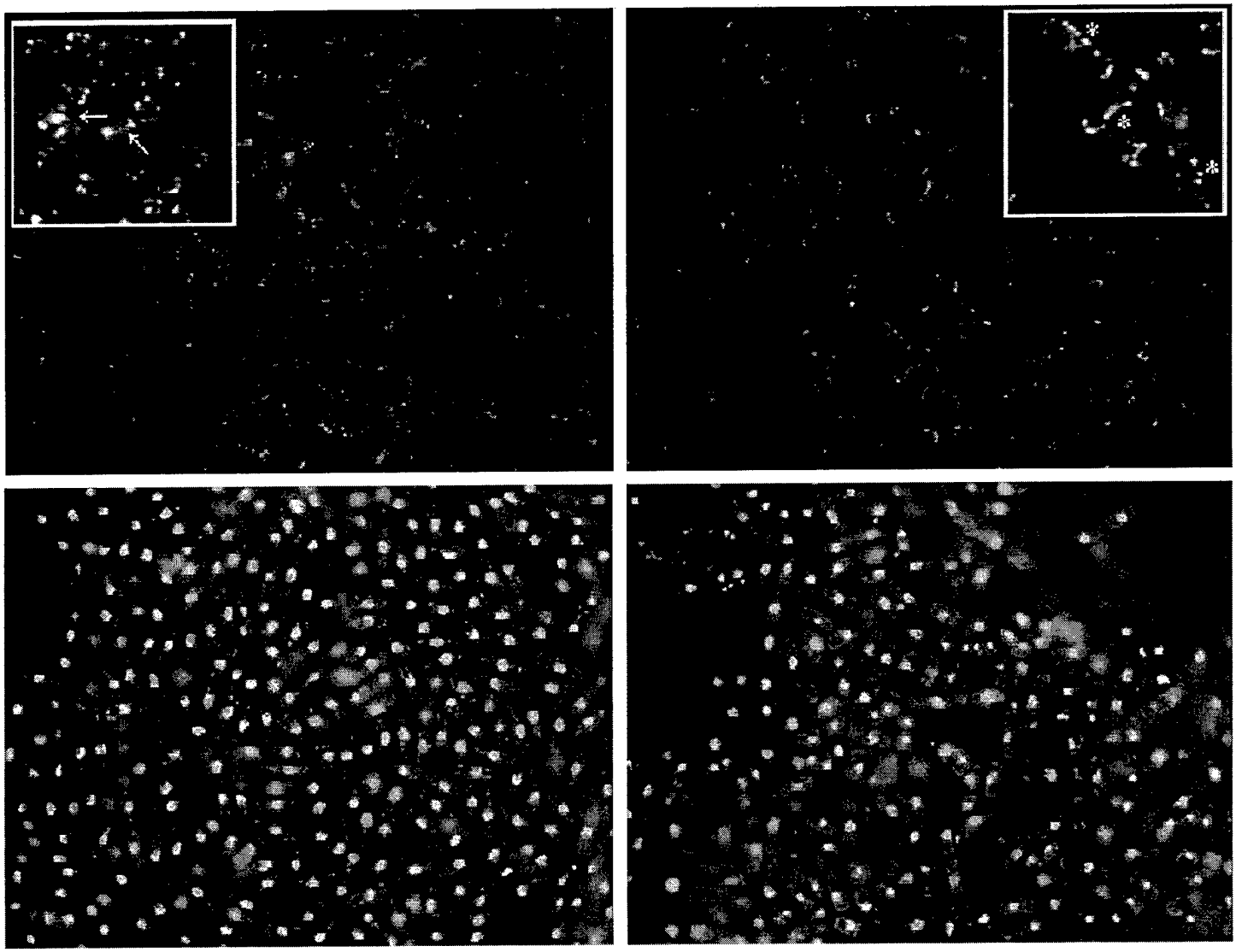


Fig. 5

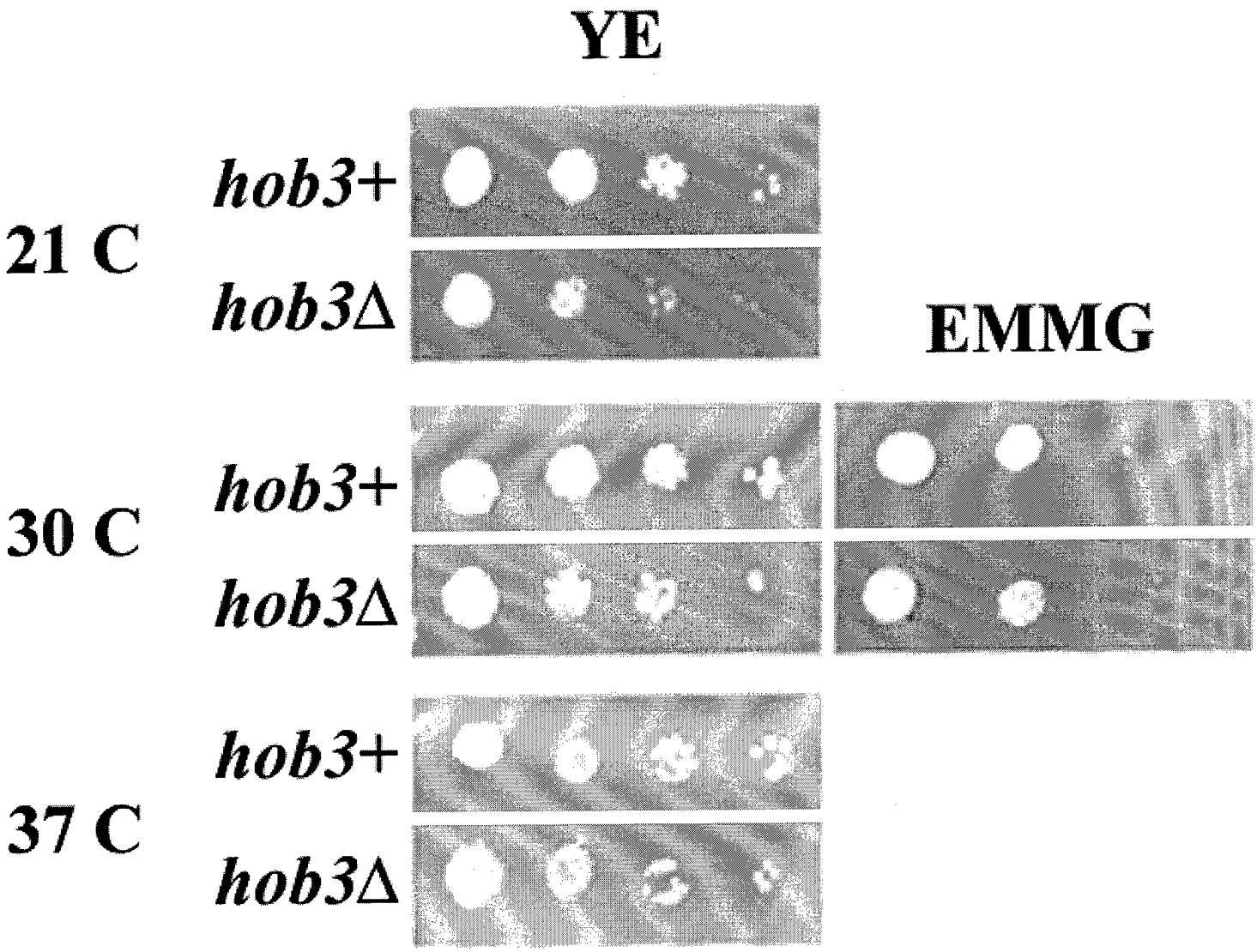


Fig. 6

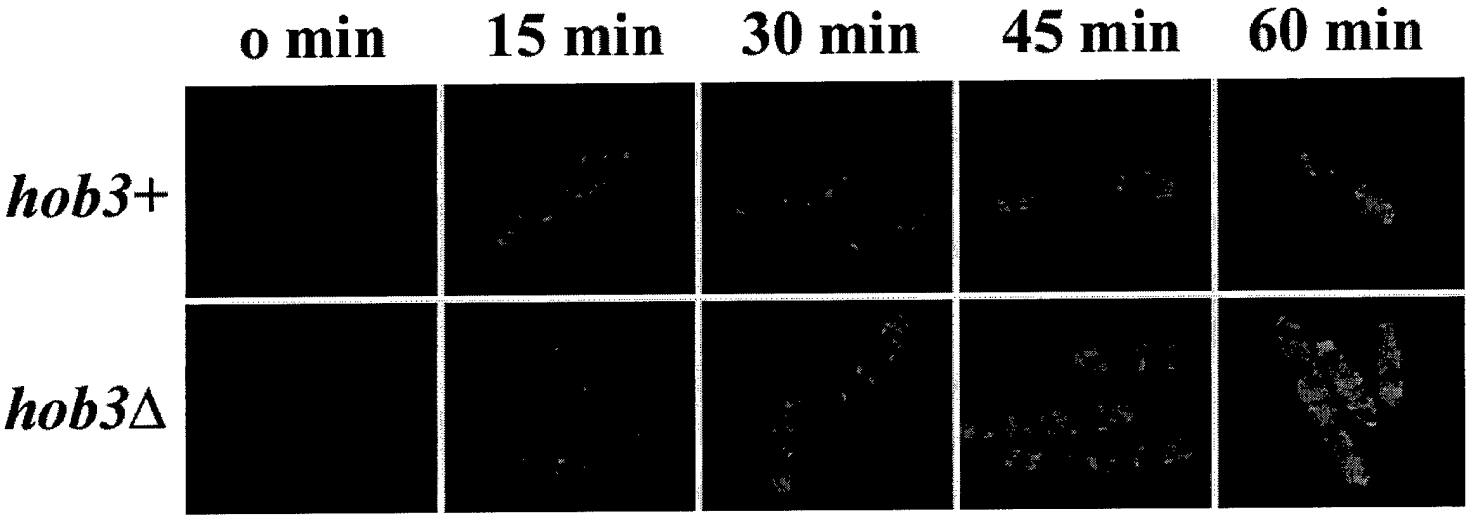
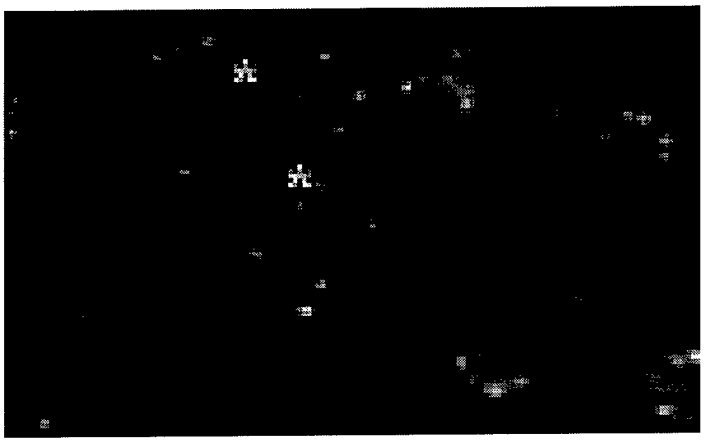
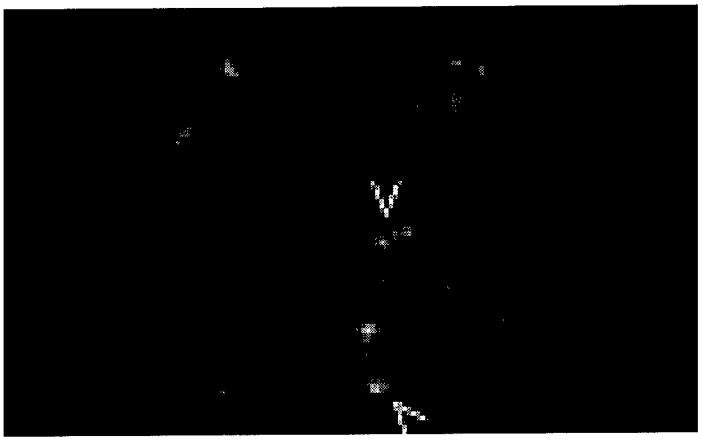


Fig. 7

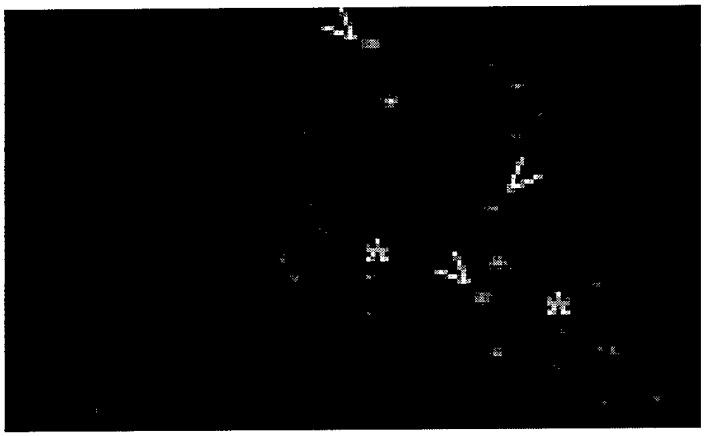
*hob3*Δ/pREP2



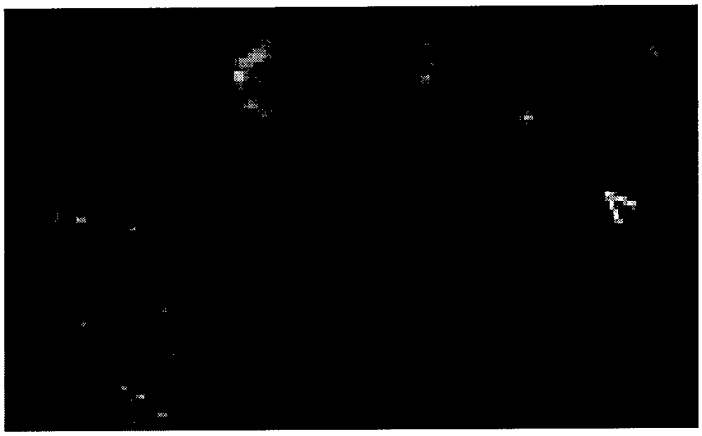
*hob3*Δ/pREP2-*hob3*+



*hob3*Δ/pREP2-RVS161

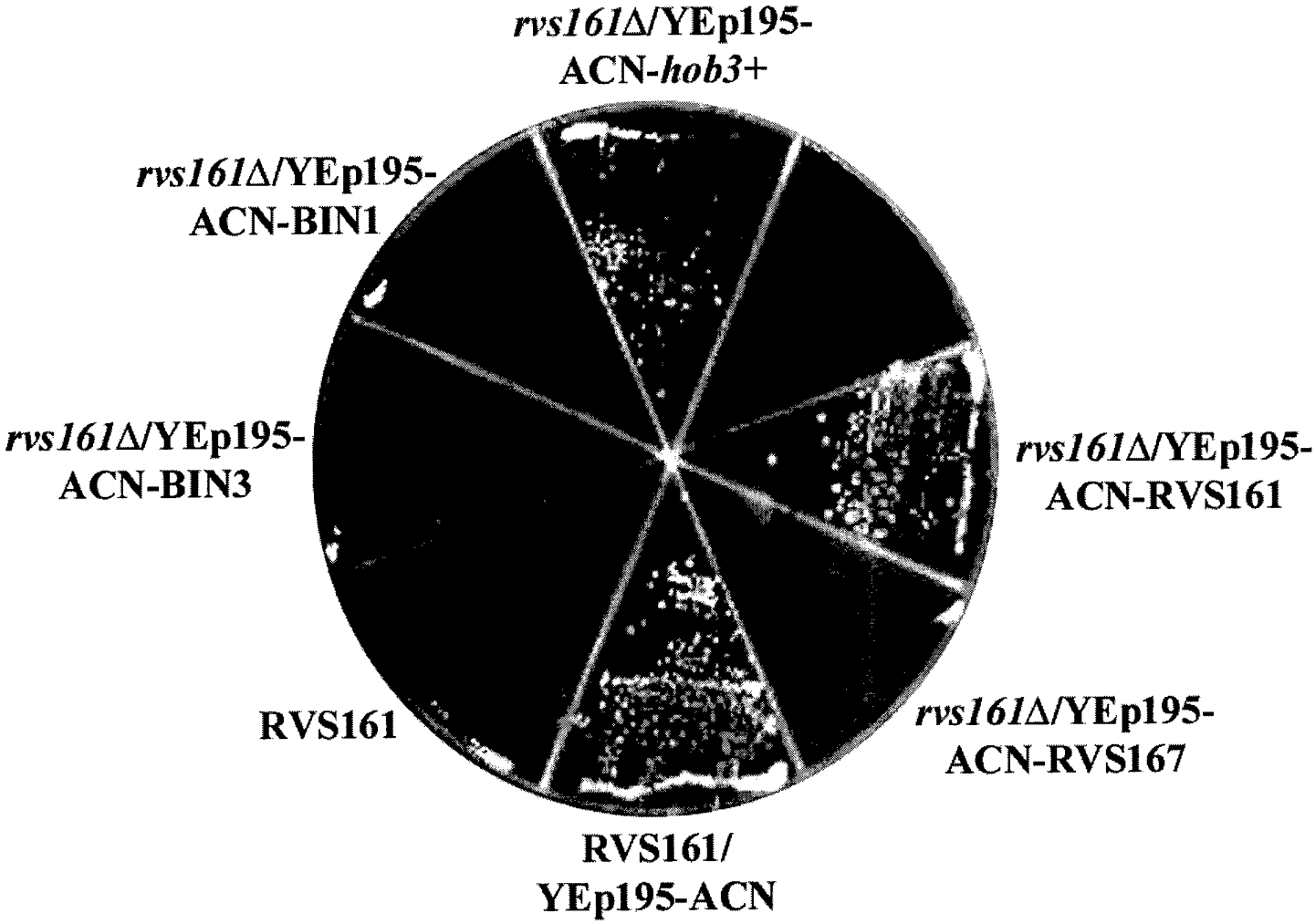


*hob3*Δ/pREP2-BIN3



*hob3*Δ/pREP2-RVS167

Fig. 8


*rvs161*Δ/YEp195-ACN-*hob3*+
*rvs161*Δ/YEp195-ACN-BIN1
*rvs161*Δ/YEp195-ACN-BIN3
*rvs161*Δ/YEp195-ACN-RVS161
RVS161
*rvs161*Δ/YEp195-ACN-RVS167
RVS161/YEp195-ACN

% Similarity	Hob3p	Rvs161p	Bin3	Bin1	Rvs167p	Amphi1	Bin2
Hob3p	-	56	29	24	31	27	26
Rvs161p		-	28	23	28	26	24
Bin3			-	27	20	22	24
Bin1				-	25	65	61
Rvs167p					-	25	29
Amphi1						-	53
Bin2							-

Table 1. Relationships between known BAR-encoding polypeptides. The BAR domains of known BAR-containing polypeptides were aligned using the GCG program GAP [59] and the percentage of identical residues were calculated for each pairwise combination. Polypeptides were truncated to 253 N-terminal amino acids for comparison.

	F-actin Distribution	1n		2n			>2n
	cortical patches	YES	NO	N/A	NO	PARTIAL	NO
	medial patches/rings	N/A	N/A	YES	NO	YES	NO
Medium	**Strain**						
YES	wild type	77% (111)	3% (4)	20% (29)			
YES	*hob3*Δ	10% (12)	61% (76)	7% (9)	6% (7)	5% (6)	8% (9)
YES/57mM NaCl	wild type	79% (73)	7% (6)	13% (12)	1% (1)		
YES/57mM NaCl	*hob3*Δ	21% (17)	22% (18)	19% (15)	10% (9)	12% (10)	15% (12)
YES/125mM NaCl	wild type	63% (10)	6% (1)	25% (4)	6% (1)		
YES/125mM NaCl	*hob3*Δ	20% (10)	44% (22)	8% (4)	16% (8)	2% (1)	10% (5)
EMM2	wild type	77% (56)	7% (5)	16% (12)			
EMM2	*hob3*Δ	26% (19)	59% (43)		7% (5)	2% (1)	7% (5)
EMM2–N	wild type	86% (64)	3% (2)	7% (6)	3% (2)		
EMM2–N	*hob3*Δ	21% (8)	31% (12)	13% (5)	21% (8)	8% (3)	7% (3)
EMM2	*hob3*Δ/ pREP2	31% (27)	49% (43)	8% (7)	6% (5)	5% (4)	
EMM2	*hob3*Δ/ pREP2-*hob3+*	49% (19)	10% (4)	26% (10)	8% (3)		
EMM2	*hob3*Δ/ pREP2-RVS161	12% (8)	63% (42)	15% (10)	7% (5)	1% (1)	
EMM2	*hob3*Δ/ pREP2-RVS167	9% (6)	60% (39)	3% (2)	14% (9)	5% (3)	5% (3)
EMM2	*hob3*Δ/ pREP2-BIN3	30% (24)	19% (15)	32% (25)	9% (7)	8% (6)	

Table 2. Quantification of F-actin distribution in *hob3*Δ mutants expressing BAR-containing polypeptides. Cells were grown to exponential phase in the medium indicated, fixed, and stained with AlexaFluor 488-phalloidin and DAPI. Cells were counted and distributed according to their nuclear content and F-actin staining pattern. The number (n) of cells analyzed is indicated in parentheses. N/A, not applicable.