Filamin (280-kDa Actin-binding Protein) Is a Caspase Substrate and Is Also Cleaved Directly by the Cytotoxic T Lymphocyte Protease Granzyme B during Apoptosis*

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We used yeast two-hybrid screening to identify the cytoskeletal protein filamin as a ligand for the proapoptotic protease granzyme B, produced by cytotoxic T lymphocytes. Filamin was directly cleaved by granzyme B when target cells were exposed to granzyme B and the lytic protein perforin, but it was also cleaved in a caspase-dependent manner following the ligation of Fas receptors. A similar pattern of filamin cleavage to polypeptides of ~110 and 95 kDa was observed in Jurkat cells killed by either mechanism. However, filamin cleavage in response to granzyme B was not inhibited by the caspase inhibitor z-Val-Ala-Asp-fluoromethylketone at concentrations that abolished DNA fragmentation. Filamin staining was redistributed from the cell membrane into the cytoplasm of Jurkat cells exposed to granzyme B and perforin and following ligation of Fas receptors, coincident with the morphological changes of apoptosis. Filamin-deficient human melanoma cells were significantly (although not completely) protected from granzyme B-mediated death compared with isogenic filamin-expressing cells, both in clonogenic survival and 51Cr release assays, whereas death from multiple other stimuli was not affected by filamin deficiency. Thus, filamin is a functionally important substrate for granzyme B, as its cleavage may account at least partly for caspase-independent cell death mediated by the granule.

Cytotoxic T lymphocytes (CTL) and natural killer (NK) cells induce target cell apoptosis through either of two contact-dependent mechanisms. The first requires a receptor-ligand interaction between a molecule of the TNF family on the surface of the killer cell and its death receptor on the target cell (1). The second mechanism operates through contact-dependent release of the granule-bound apoptotic mediators perforin and granzyme B (grB) and is chiefly responsible for eliminating virus-infected cells (2).

Fas ligation leads to the recruitment of a death-signaling complex at the inner leaflet of the cell membrane (3). This results in activation of an evolutionarily conserved, caspase-dependent cell death pathway present in virtually every cell. The subsequent cleavage of vital proteins and DNA leads to cell death. The precise mechanism of collaboration between perforin and grB is unknown; it is generally accepted that perforin, a pore-forming protein, enables proapoptotic serine proteases (granzymes) to access their substrates in the target cell, cleavage of which leads to apoptosis (4–6). Thus, perforin deficiency inactivates the killing mechanisms that operate through granzymes (7, 8). Although several different granzymes are capable of inducing cell death in a perforin-dependent manner (9), the most potent and best characterized is grB, with the ability to cleave proteins after specific Asp residues that enables it to directly activate procaspases, commencing with the cleavage of procaspase-3 (10). The congenital absence of grB from the CTL of mice delays target cell apoptosis and is manifested in their reduced ability to provoke nuclear damage including DNA fragmentation (11).

Although caspase activation is clearly an important function of grB, we (12) and others (13) have shown that grB-mediated apoptosis can occur despite the absence of active caspases. However, the molecular targets responsible for caspase-independent death stimulated by grB have so far proven elusive. The cumulative data from several laboratories have indicated strongly that additional pathways to target cell death arose to overcome the effects of viral anti-apoptotic molecules that delay the suicide of infected cells by blocking caspases (14). These antiviral pathways probably represent the cumulative response to viral strategies that delay apoptosis, which have coevolved with the immune system (14). Among the many viral apoptosis antagonists identified, prototypes include the cowpox protein CrmA (15) and baculovirus p35 (16), both direct caspase inhibitors.

Cell death in the absence of active caspases implies that other proteases can cleave key proteins in the target cell. We have previously reported that grB is rapidly targeted to the nucleus of target cells (17–20), suggesting that grB might induce nuclear collapse when caspases are inactive (21, 22). GrB can directly cleave some nuclear proteins such as nuclear matrix antigen and poly(ADP-ribose) polymerase (22), and granzyme A can activate DNA fragmentation in a caspase-independent manner in cell extracts (23). We also recently demonstrated a marked reduction of nuclear apoptotic changes in response to granzyme B when caspases were inactivated; however, the cells still died through a caspase-independent pathway manifested by cytoplasmic and cell membrane damage (12). These non-nuclear events were a reliable predictor of cell death, as they correlated closely with colony numbers in
long-term clonogenic assays (12). In another study in which multiple parameters of CTL-mediated apoptosis were examined, non-nuclear manifestations such as phosphosserine externalization, cell membrane blebbing, and changes in mitochondrial membrane potential did not require active caspases, whereas nuclear changes such as chromatin condensation and DNA fragmentation did (24). It was recently reported that grB can induce activation of caspase-activated DNase (CAD) by cleaving its inhibitor (ICAD), thus leading to DNA fragmentation (25). Some direct activation of CAD-induced DNA damage persisted despite caspase inhibition; however, the concentrations of grB used was 40–80 times higher than needed to induce grB-mediated apoptosis, so the physiological significance of this pathway remains in question. Moreover, the nucleus is dispensable for CTL-mediated cell death (26), indicating that damage to other parts of the cell are more crucial for determining apoptotic outcome.

These results strongly suggested that direct cleavage of non-nuclear substrates by granzymes can lead to caspase-independent cell death. However, no direct granzyme substrates (other than the caspases and ICAD) have been described outside the nucleus. To shed light on unidentified grB substrates, we used yeast two-hybrid screening to identify the cytoskeletal protein filamin (280-kDa actin-binding protein, ABP-280) as a ligand for grB. Herein we demonstrate that filamin can be directly cleaved by grB independently of caspase activation and can also be cleaved following Fas ligation in a caspase-dependent manner. Cells deficient in wild-type filamin are less sensitive to grB-mediated cell death, indicating that filamin is a candidate for the mediation of a caspase-independent non-nuclear pathway to apoptosis in response to grB.

MATERIALS AND METHODS

Cell Culture—The human leukemia cell line Jurkat was maintained in RPMI medium supplemented with 10% fetal calf serum at 37 °C in air containing 5% CO2. The human melanoma cell line M2, which lacks expression of filamin mRNA, and the isogenic cell line A7, in which expression was re-established by gene transfection, were maintained as above. Both cell lines were a kind gift of Dr. John Hartwig (Harvard Medical School).

Chemicals and Reagents—Human perforin, purified as described, was kindly supplied by Dr. Chris Froelich, Evanston University, Evanston, IL (27). Pneumococcal pneumolysin (PLO) (a gift from Dr. James Paton, University of Adelaide, Australia) was activated in phosphate-buffered saline containing 2-mercaptoethanol for 30 min. at room temperature. A sublytic dose of the membranolytic agents was defined as producing <10% specific release of 3 Cr in a 4-h assay at 37 °C. Neither membranolytic agent was inhibitory for the Asp-ase activity of grB, nor was membrane perforation negatively affected by the caspase inhibitors described below (data not shown). Immunoaffinity purification of human grB from nuclear lysates of YT (human NK leukemia) cells was performed as described (28). The grB was free of grA and Met-ase activities, as well as perforin, as demonstrated by Western blotting and functional assays (28). The oligopeptide caspase inhibitors z-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk) and Phe-Ala-fluoromethylketone (z-FA-fmk) were purchased from Enzyme Systems Products (San Diego, CA), dissolved in Me2SO, and stored in aliquots at −20 °C. Final concentrations of Me2SO did not exceed 0.5% in any of the assays. Polyclonal antisera detecting human ABP-280 were raised in rabbits immunized with a fusion protein consisting of glutathione S-transferase and the C-terminal 476 amino acids of filamin, which was purified by chromatography on glutathione-agarose.

Yeast Two-hybrid Screening—The cDNA encoding human grB was the template in a plasmid vector pGAD10 (CLONTECH). Clones with putative interactions between bait and prey were selected by growth on medium lacking Trp/Leu/Arg (TLA) and Trp/Leu/His (TLH). DNA from clones of interest was used to transform electrocompetent Escherichia coli TOP10F cells, and cDNA inserts of interest were sequenced.

Asays of Apoptosis—125 Cr release (a measure of non-nuclear cell damage) and TUNEL assays were performed as described (12). Fas-mediated apoptosis of Jurkat cells was accomplished by incubating the cells in the presence of CH11 anti-Fas antibody (100 ng/ml) for 4 h at 37 °C. Cells undergoing apoptosis in response to anti-Fas antibody or perforin and grB were analyzed by confocal laser scanning microscopy, as described previously in detail (17–19). M2 and A7 cells were induced to undergo apoptosis by exposure to medium containing staurosporine for 24 h. Loss of viability was assessed by trypan blue exclusion.

Asays of Clonogenic Survival—M2 and A7 cells in logarithmic growth phase were incubated for 2 h with a sublytic quantity of porin alone and/or with grB (30–120 nM). Cells (5 μl from a stock of 4 × 104 cells/ml) treated with the various apoptotic stimuli were plated out in triplicate in individual wells of a 48-well plate and then incubated at 37 °C for 4–5 days when discrete colonies were counted under an inverted microscope.

RESULTS

The C Terminus of Filamin Contains a grB Binding Site—To identify potential new ligands for grB, we expressed mature human grB as a chimeric protein with the GAL4 DNA binding domain for yeast two-hybrid studies. The same approach was successful in identifying substrates of caspases (apoptotic cysteine proteases) (29). As in the previous study, we engineered an amino acid substitution at the active site residue (Ser to Ala) to favor more stable protease-substrate interactions. Inactive variants of grA have also been used to purify its putative ligands (30). The mutated grB was coexpressed in yeast cells with a library of proteins expressed as fusions with the GAL4 transcription activation domain. Serial rounds of screening identified clones in which growth on selective media suggested the presence of bona fide interactors for grB. Sequencing revealed that one clone encoded in frame the 476 amino acids (residues 2172–2647) at the C terminus of the cytoskeletal protein, filamin (ABP-280). The interaction with grB was specific, as the filamin domain did not bind to control proteins p53, CD46, or IFI 16 (data not shown). Filamin is a ubiquitous phosphoprotein located in the peripheral cytoplasm, where its N terminus binds to and promotes orthogonal branching of actin filaments (31), contributing to membrane stability and the ability to withstand mechanical stress (32) and enabling cell migration (33).

Filamin Is Cleaved during Apoptosis Induced through Fas Ligation and by grB—The C-terminal 476 amino acids of human filamin contains 16 Asp residues, but none conformed closely to a tetrapeptide consensus sequence for preferred cleavage by grB or caspases (34, 35). To establish whether filamin is cleaved during apoptosis induced by grB, or when cells are killed through Fas ligation (the two mechanisms used by cytotoxic lymphocytes), lysates from dying Jurkat cells were probed in Western blots with an antiserum raised against the filamin domain did not bind to control proteins p53, CD46, or IFI 16 (data not shown). Filamin is a ubiquitous phosphoprotein located in the peripheral cytoplasm, where its N terminus binds to and promotes orthogonal branching of actin filaments (31), contributing to membrane stability and the ability to withstand mechanical stress (32) and enabling cell migration (33).

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Filamin cleavage was seen; however, the cleavage was not blocked by z-VAD-fmk (Fig. 1). In contrast, this concentration of z-VAD-fmk completely abolished DNA fragmentation as measured in the TUNEL assay and cleavage of poly(ADP-ribose) polymerase (see Refs. 4 and 12, and data not shown). Indeed, grB-mediated cleavage of filamin was not inhibited by pretreatment of the cells with concentrations of z-VAD-fmk up to 50 μM, and filamin cleavage also persisted in MCF-7 human breast cancer cells, which lack caspase-3 (data not shown). Proteolytically inactive (in) grB (with its the active site Ser residue mutated to Ala) expressed in baculovirus-infected insect SF21 cells was unable to induce filamin cleavage (Fig. 1) or cell death (data not shown), indicating the need for Asp-ase activity for both phenomena. When immunoprecipitated filamin was incubated with purified grB, filamin was cleaved into a number of peptides (Fig. 2), confirming that grB can directly cleave filamin in the absence of other proteases. Several polypeptides (205, 200, and 110 kDa) were of similar size to those observed after intact cells were incubated with perforin and grB. However, the 95-kDa polypeptide seen when intact cells were exposed to perforin/grB was not observed. Collectively, our results suggested that (i) certain sites in filamin are cleaved far more efficiently by grB than are others, and (ii) proteases other than grB may also contribute to filamin processing in the absence of caspases. Filamin is therefore the target of several classes of protease during apoptotic death.

Intracellular Redistribution of Filamin Is Coincident with Cell Death Induced by Perforin and grB—We next used confocal microscopy to examine the subcellular distribution of filamin in Jurkat cells undergoing apoptosis in response to grB. Filamin is normally located subjacent to the plasma membrane, where it binds transmembrane proteins including the β2-integrin CD18 (36) or the platelet glycoprotein GpIb/9 (37), linking the plasma membrane and the actin cytoskeleton. Cells were exposed either to anti-Fas antibody for 4 h or to perforin (Pfp) or the bacterial pore-forming toxin, PLO, alone or in combination with grB for 2 h (Fig. 3). Like perforin, PLO can also deliver grB to induce apoptosis in target cells (4). In cells that were mock-treated or exposed to Pfp or PLO alone (Fig. 3, left) or to grB alone (data not shown), filamin staining was seen only immediately adjacent to the cell membrane or within pseudopodal membrane projections. In each of these controls, the Jurkat cells remained free from apoptotic changes. However, the filamin staining pattern was markedly altered in cells exposed to both the Pfp/PLO and active grB or to anti-Fas antibody (Fig. 3, right). Filamin became dissociated from the membrane, and was diffusely distributed through the cytoplasm of the dying cells. Filamin continued to be excluded from the nucleus, causing the outlines of apoptotic nuclei to become easily seen. Many of the dying cells also demonstrated the cytoplasmic shrinkage characteristic of apoptotic death.

Deficiency of Filamin Is Associated with Resistance to grB-mediated Apoptosis—In previous work, we postulated that caspase-independent pathways to apoptosis are likely to be localized in the cytoplasm (12, 14). Our demonstration that filamin cleavage is not dependent on caspases raised the possibility that filamin is part of a non-nuclear pathway to cell death mediated by grB, prompting us to evaluate the effect of filamin cleavage on cell survival. The only cells suitable (and available) for such a comparison are a pair of nonclonal isogenic human melanoma cell lines, A7 and M2, that have long served as the best available model of functional filamin deficiency (Refs. 38 and 39).2 M2 cells fail to synthesize filamin and have no filamin mRNA; however, expression has been re-established in A7 cells by gene-mediated transfection, resulting in approx-
Filamin Is a Caspase-independent, Non-nuclear Cell Death Substrate of grB—Filamin is the first non-nuclear substrate of grB identified whose direct cleavage influences cell survival. Although filamin is a substrate for caspases, grB can also cleave filamin directly when caspases are inactivated. Under most circumstances, grB is a powerful apoptotic mediator as it can efficiently activate the caspase cascade, commencing with the cleavage of procaspase-3 (10). However, cell death through grB still occurs when caspases are inactivated by caspase inhibitors such as p35 or by z-VAD-fmk. Interestingly, commitment to death under these circumstances is accompanied by minimal initial nuclear damage, consistent with the fact that nuclear events are dispensable for CTL-mediated cell death (26). In contrast, non-nuclear manifestations of cell death persist despite caspase inactivation; and despite their normal nuclear morphology early in apoptosis, the cells do not survive in clonogenic assays, indicating they are already destined to die (12). We have therefore favored the likelihood that cytoplasmic, not nuclear, substrates are crucial in delivering the lethal hit from grB. Our present findings that cleavage of a cytoplasmic cytoskeletal element, filamin, can influence cell survival in response to grB is consistent with this hypothesis.

Cytoskeletal Elements Can Be Critical Substrates for Regulating Cell Death—Although the mechanism by which filamin deficiency protects cells against grB is not yet understood at the molecular level, there are several other instances of the cytoskeleton controlling apoptotic outcome. For example, the light chain of the dynein motor complex sequesters the pro-apoptotic BH3-only Bcl-2 family member, Bim, until an apoptotic stimulus triggers Bim release, thus permitting it to access and disrupt the integrity of the mitochondrial membrane (40). By analogy, filamin might sequester a key grB substrate or even grB itself by direct binding; however, the prediction of this model is that the levels of a free grB should increase in M2 cells, thereby increasing their susceptibility to apoptosis. This prediction is clearly at odds with our findings, which, if anything, predict the opposite scenario, that binding of grB to filamin might potentiate its ability to bind and cleave other proapoptotic substrates. An alternative possibility is that toxic cleavage products may be generated from filamin by grB and possibly the caspases. This mechanism would be reminiscent of the cleavage of another actin-binding protein, gelsolin, by caspases (41). Upon cleavage by caspase-3, it has been proposed that a 41-kDa N-terminal fragment of gelsolin induces cleavage of F-actin filaments, leading to cytoskeletal collapse (42). However, this mechanism remains controversial, as gelsolin overexpression did not result in increased sensitivity of lymphocytes to apoptotic stimuli (43). To date, there is no evidence that filamin cleavage products are toxic, but if this were the case one would expect M2 cells to also be resistant to receptor-mediated killing, as Fas ligation also resulted in filamin cleavage in Jurkat cells (Fig. 1). Unfortunately, M2 and A7 cells are resistant to Fas- and TNF-mediated cell death, making it impossible to test this prediction. We are currently mapping filamin cleavage sites and testing whether the cleavage products are toxic.

The findings presented in this paper represent a consider-
able step forward in our understanding of the impressive re-
dundancy in proapoptotic mechanisms within cytolytic gran-
ules. We have defined a novel pathway used by grB to overcome 
potential inhibition of caspase-mediated cell death pathways, 
involving the proteolysis of a cytoskeletal protein, filamin, the 
 cleavage of which influences cell survival. The ability of grB to 
directly induce cell death in the cytoplasm without invoking 
caspase-mediated nuclear damage represents an evolutionary 
adaptation of the immune system to counter the ability of 
viruses to block the intrinsic cell suicide pathway.

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REFERENCES

1. Rouvier, E., Luciani, M. F., and Golstein, P. (1993) J. Exp. Med. 177, 195–200
2. Trapani, J. A., Jones, D. A., and Sutton, V. R. (1998) Springer Semin. Immuno-
pathol. 19, 323–344
3. Boldin, M. P., Golcharov, T. M., Yoltsev, Y. T., and Wallach, D. (1996) Cell 85, 
813–825
4. Browne, K. A., Blink, E., Sutton, V. R., Froelich, C. J., Jans, D. A., and 
Trapani, J. A. (1999) Mol. Cell. Biol. 19, 8604–8615
5. Froelich, C. J., Orth, K., Turbow, J., Seth, P., Gottlieb, R., Bahler, B., Shah, 
G. M., Bleackley, R. C., Dixit, V., and Hanna W. (1996) J. Biol. Chem. 271, 
29073–29079
6. Froelich, C. J., Dixit, V. M., and Yang, X. (1998) Immunol. Today 19, 30–36
7. Kagi, D., Ledermann, B., Burki, K., Seiler, P., Odermatt, B., Olsen, R. J., 
Podack, E. R., Zinkernagel, R. M., and Hengartner, H. (1994) Nature 369, 
31–37
8. Kagi D., Ledermann, B., Burki, K., Hengartner, M., and Zinkernagel, R. M. 
(1994) Eur. J. Immunol. 24, 3068–3072
9. Shi, L., Kam, C.-M., Powers, J. C., Aebersold, R., and Greenberg, A. H. (1992) 
J. Exp. Med. 176, 1521–1529
10. Yang X., Stennicke, H. R., Wang, B., Green, D. R., Janicke, R. U., Sririvasan, 
R., Seth, P., Salvesen, G. S., and Froelich, C. J. (1998) J. Biol. Chem. 273, 
34278–34283
11. Hensel, J. W., Wesselschmidt, R. L., Shresta, S., Russell J. H., and Ley, T. J. 
(1994) Cell 76, 977–987
12. Trapani, J. A., Jones, D. A., Browne, K. A., Smyth, M. J., Jans, P. F., and Sutton, 
V. R. (1998) J. Biol. Chem. 273, 27934–27938
13. Sarin, A., Williams, M. S., Alexander-Miller, M. A., Berzofsky, J. A., 
Zacharchuk, C. M., and Henkart, P. A. (1997) Immunity 6, 209–215
14. Trapani, J. A., Sutton, V. R., and Smyth, M. (1999) Immunol. Today 20, 
351–356
15. Tewari, M., and Dixit, V. M. (1995) J. Biol. Chem. 270, 3255–3260
16. Clem, R. J., Fechheimer, M., and Miller, L. K. (1991) Science 254, 1388–1390
17. Jans, D. A., Jones, P., Briggs, L. J., Sutton V. R., and Trapani, J. A. (1996) 
J. Biol. Chem. 271, 30781–30789
18. Jans, D. A., Briggs, L. J., Jones, P., Froelich, C. J., Parasivam, G., Williams, 
E. A., Kumar, S., Sutton V. R., and Trapani, J. A. (1998) J. Cell Sci. 111, 
2645–2654
19. Trapani, J. A., Browne, K. A., Smyth M. J., and Jans D. A. (1996) J. Biol. 
Chem. 271, 4127–4133
20. Trapani, J. A., Jans, P., Smyth, M. J., Froelich, C. J., Williams, E. A., Sutton, 
V. R., and Jans, D. (1998) Cell Death Differ. 5, 488–496
21. Andreu, F., Roy, S., Thornberry, N., Rosen, A., and Caccialosa, L. (1998) 
Immunity 8, 451–460
22. Froelich C. J., Hanna, W. L., Poirier, G. G., Duriez, P. P., D’Amours D., 
Salvesen G. S., Alnemri E. S., and Shah, G. M. (1996) Biochem. 
Biophys. Res. Commun. 227, 658–665
23. Beresford P. J., Xia, Z., Greenberg A. H., and Leberman J. (1999) Immunity 
10, 585–594
24. Sarin A., Haddad E. K., and Henkart, P. A. (1998) J. Immunol. 161, 
2810–2816
25. Thomas, D. A., Du, C., Xu, M., Wang, X., and Ley, Y. J. (2000) Immunity 
12, 621–632
26. Nakajima, H., Golstein, P., and Henkart, P. A. (1995) J. Exp. Med. 185, 
1965–1910
27. Froelich, C. J., Turbow J., and Hanna, W. (1996) Biochem. Biophys. Res. 
Commun. 222, 44–49
28. Trapani, J. A., Browne, K. A., Dawson, M., and Smyth, M. J. (1993) Biochem. 
Biophys. Res. Commun. 195, 910–920
29. Kamada S., Kusano, H., Fujita, H., Ohitsu, M., Koya, R., Kuzumaki, K., and 
Tsujimoto, Y. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 8532–8537
30. Beresford P. J., Kam, C.-M., Powers, J. C., and Leberman, J. (1997) Proc. Natl. 
Acad. Sci. U. S. A. 94, 9285–9290
31. Gorlin, J. B., Yamin, R., Egan S., Stewart, M., Stossel, T. P., Kwiatkowski, M., 
and Hartwig, J. H. (1990) J. Cell Biol. 111, 1089–1105
32. Glogauer M., Arora, P., Chou, D., Jannmez, P. A., Downey, G. P., 
and McCulloch, C. A. (1998) J. Biol. Chem. 273, 1689–1698
33. Fox, J. W., Lamperti, P. D., Eksioglu, Y. Z., Hong, S. E. W., Feng, Y., Graham, 
I. E. Scheffer, I. E., Dobyns, W. B., Hirsh, B. A., Radtke, R. A., Berkovic, 
S. F., Bullrich, F., Fritz, L. C., Trapani, J. A., Tomasselli, K., Litwack, G., and 
Garcia-Calvo, M., Houtzager, M., Nordstrom, P. A., Roy, V., Vaillancourt, 
J. P., Chapman, K. T., and Nicholson, D. W. (1997) J. Biol. Chem. 272, 
17907–17911
34. Sharma, C. P., Ezzell, R. M., and Arnaut, A. A. (1995) J. Immunol. 154, 
3461–3470
35. Shi, L., Kraut, R. P., Aebersold, R., and Greenberg, A. H. (1992) J. Exp. Med. 
175, 553–566
36. Cunningham, C. C. (1995) J. Cell Biol. 129, 1589–1599
37. Cunningham, C. C., Gorlin, E., Kwiatkowski, D. J., Hartwig, J. H., Jannmez, 
P. A., Byers, H. R., and Stossel, T. P. (1992) Science 255, 325–327
38. Puthalakath H., Huang, D. C., O'Reilly, L. A., King, S. M., and Strasser, A. 
(1999) Mol Cell 3, 287–296
39. Kothakota S., Azuma, T., Reinhard, C., Klippel, A., Tang, J., Chu, K., 
McGarry, T. J., Kirschner, M. W., Koths, K., Kwiatkowski D. J., and 
Williams, L. T. (1997) Science 278, 294–298
40. Kwiatkowski, D. J. (1999) Curr. Opin. Cell Biol. 11, 103–108
41. Posey, S. C., Martelli, M. P., Azuma, T., Kwiatkowski, D. J., and Bierer, B. E. 
(2000) Blood 95, 3483–3488

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