A Novel F Box Protein, NFB42, Is Highly Enriched in Neurons and Induces Growth Arrest*

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NFB42 (neural F Box 42 kDa) is a novel gene product that is highly enriched in the nervous system. Its predicted protein contains an F box, a motif recently shown to couple cell cycle regulation to the proteasome pathway (Bai, C., Sen, P., Hofmann, K., Ma, L., Goebel, M., Harper, J. W., and Elledge, S. (1996) Cell 86, 263–274). NFB42 mRNA and protein are expressed in all major areas of the adult rat brain but are not detected in non-neural tissues. NFB42 protein is localized primarily to the cytoplasm of neurons and does not appear to be present in glia. The presence of an F box in NFB42 suggests that it may be involved in cell cycle regulation; however, its expression in postmitotic neurons indicates that it is not involved in regulating typical cell cycle events. In an initial attempt to characterize the function of this protein, NFB42 was transfected into N1E-115 neuroblastoma and Chinese hamster ovary cells. The expression of full-length NFB42, but not an F box deletion mutant, inhibits proliferation in both cell lines. Additional experiments demonstrate that NFB42 interacts with Skp1p, a component of the proteasome pathway, and deletion of the F box also inhibits this interaction. Overall, the expression pattern of NFB42, along with the presence of an F box domain and the ability to inhibit growth, suggests that it may play a role in maintaining neurons in a postmitotic state.

Cellular and biochemical mechanisms responsible for maintaining neurons in a permanently postmitotic state are poorly understood; however, processes known to be involved in regulating the cell cycle, including the modulation of phosphorylation, inhibition of cyclin-dependent kinases (cdks)1 by cdk inhibitors, and changes in message levels of cell cycle-associated proteins, are all thought to be involved in the growth arrest accompanying the terminal differentiation of neurons (1–6). It is likely that additional regulatory proteins and pathways are activated to help maintain neurons in a permanent noncycling state and to protect against lethal attempts to reenter the cell cycle. Strong proliferative stimuli can lead to the initiation of cell death in postmitotic neurons (7); therefore, the presence of additional control mechanisms seems particularly critical, given that neurons maintain many of the signal transduction pathways that couple extracellular signals to control the cell cycle in proliferating cells (8–10). Neurons also express a number of potential regulators of the cell cycle, such as cyclins and cdks, which function in the control of normal cellular processes independent of proliferation (6, 11–13).

In addition to post-translational modifications, expression of cdk inhibitors, and modulation of transcription, an additional level of cell cycle regulation is accomplished through the targeted degradation of proteins (14–16). The degradation of regulatory proteins is known to be involved in the control of cell cycle entry, progression, and exit (14, 16). Recently, key proteins coupling cell cycle regulation with ubiquitin-dependent proteolysis have been identified in yeast. For example, G1/S phase transition and entry into the cell cycle require degradation of the inhibitor Sic1p. The degradation process is initiated when Sic1p becomes phosphorylated by the G1 cyclin-dependent kinase Cln/Cdc28, leading to its recognition and binding by Cdc4p, which in turn binds Skp1p through an F box motif (17, 18–20). F box-containing proteins like Cdc4p make up part of an E3 ubiquitin ligase complex involved in targeting proteins for destruction via the ubiquitin-proteasome pathway (18, 19). Therefore, F box proteins can recognize targets to be degraded and link the targeted protein to the ubiquitin ligase complex through their ability to bind Skp1p. The degradation of G1 cyclins (19) and p58, a regulatory component of the kinetochore (21, 22) is also controlled by a Skp1p/F box protein-dependent mechanism. In addition, Skp1p may perform regulatory functions in these processes by binding kinases and directing them to their F box-containing targets (22).

Although the importance of Skp1p in G1/S phase transition and kinetochore function is clear in Saccharomyces cerevisiae, little is known about its function in mammalian cells. Along with Skp2p and p9, it replaces PCNA/p21WAF1 in the cyclin A/cdk2 complex in many transformed cells (23). Skp1p has also been shown to be expressed in postmitotic neurons (24). Even less is known about mammalian F box-containing proteins, although cyclin F and Skp2p contain this motif, and both are thought to modulate cell cycle function (17, 25).

In the present study, we describe the cloning and initial characterization of a novel F box-containing protein that is highly enriched in postmitotic neurons. The expression of this protein, NFB42, inhibits proliferation in both neural and non-neural cells, which is consistent with a possible function of
helping to maintain neurons in a postmitotic state and/or protecting neurons from an attempted reentry into the cell cycle.

EXPERIMENTAL PROCEDURES
cDNA Cloning—RNA isolated from differentiated PC12 cells after nerve growth factor removal was used to make a ZAP cDNA library (Stratagene Cloning Systems, La Jolla, CA). A 0.7-kb cDNA representing the 3′ end of NFB42 was isolated from the library in a differential screen (control versus nerve growth factor removed for 12 h) to identify messages induced during apoptosis (12 h was the point at which approximately 50% of the cells had committed to undergo programmed cell death). Several full-length clones were isolated using conventional phage screening techniques and the 0.7-kb fragment as a probe. Sequencing was performed using heat-denatured double-stranded plasmids, 32P-labeled DATP, and Sequenase (United States Biochemical Corp., Cleveland, OH). GC-rich areas of the cDNA were sequenced using: 1) double-stranded plasmids denatured both chemically and by heat, 2) single-stranded plasmids generated from Bluescript Phagemid (26), 3) 7-deaza-dGTP DNA sequencing (United States Biochemical Corp.), and 4) gels run at high constant power settings to achieve plate temperatures from 55 °C to 65 °C.

Northern Blots—Total RNA was isolated from adult rat tissues using the guanidinium method of Chomczynski and Sacchi (27) and polyadenylated RNA was isolated using oligo(dT)25-cellulose (Invitrogen, Carlsbad, CA). Polyadenylated RNA samples (1 μg) were separated by electrophoresis on an agarose-formaldehyde gel and transferred to nylon membranes, followed by UV cross-linking. Membranes were prehybridized for 2 h at 42 °C in 50% formamide, 5× saline/ sodium phosphate/EDTA, 1% SDS, 5× Denhardt's solution, 100 μg/ml sheared denatured salmon sperm DNA, and 2.25× deionized water, followed by incubation with [32P]cDNA probe (106 cpm/ml) made by random primer extension of a 662-bp fragment representing the 3′ end of NFB42 (nucleotides 634–1296). Filters were washed with 2× saline/sodium phosphate/EDTA and 0.1% SDS at 65 °C for 30 min, with washed with 0.2× saline/sodium phosphate/EDTA or 0.1% SDS at 65 °C for 5–30 min, and exposed to x-ray film at −70°C.

Interaction of NFB42 with Skp1p—To determine the interaction between NFB42 and Skp1p, the 0.7-kb fragment was used as a probe to screen a yeast two-hybrid expression construct (3:1 ratio) into CHO cells with SuperFect Transfection Reagent (Qiagen, Inc., Valencia, CA) following the manufacturer's instructions. At 16 h post-transfection, the cells were split into 35-mm dishes (approximately 1.0–3.0 × 106 cells/dish). 2 h later, GFP-positive cells were counted in three dishes (five random fields/dish; time 0). Approximately 100–300 cells were counted in each dish at time 0. GFP-positive cells were visualized using a Nikon Diaphot inverted microscope equipped with a mercury vapor light source (excitation filters, 450–490 nm). Subsequent time points were counted in the same random fields selected at time 0. For proliferation of the entire population, vector-transfected populations were counted using phase light microscopy (i.e. 90% nontransfected/10% transfected cells). Data are expressed as the means ± S.E. from three independent experiments.

RESULTS
NF2B42 was initially isolated from a differential screen to identify cDNAs induced after the removal of nerve growth factor. In initial experiments, the NF2B42 message was increased 4–6-fold...
after the removal of nerve growth factor (data not shown); however, subsequent studies indicated that the message was not consistently induced. Initial characterization and tissue distribution of NFB42 mRNA indicated that it was novel and highly enriched in the brain, which warranted further investigation into its possible function in the nervous system.

Sequence information from several NFB42 cDNAs indicate that its message is GC rich (62%), has a short 64-nucleotide 5' untranslated region followed by a consensus Kozak start site, and open reading frame coding for a predicted 296-amino acid acidic protein (nucleotides 65–952). This is followed by a 344-nucleotide 3' untranslated region, including a polyadenylation signal (AATAAA) at nucleotides 1254–1259 (Fig. 1A). Sequence analysis of the predicted NFB42 protein shows several motifs and homologies to previously described proteins. The highest homology is to two peptide fragments sequenced from one of the most abundant proteins in the organ of Corti, OCP1 (30). Given the high homology between rat NFB42 and guinea pig OCP1 (84% identity when comparing the two peptides totaling 67 amino acids), these may represent the same protein.

Three different areas of NFB42 (amino acids 74–92, 157–226, and 250–289) show significant homology to Caenorhabditis elegans clone C14B1.3, suggesting that these may be homologues or members of the same family. C14B1.3 was isolated...
plasticity of pyramidal neurons). The presence of an F box in NFB42 suggests that it may be involved in cell cycle regulation; however, its localization to postmitotic neurons suggests that it is not involved in regulating typical cell cycle processes. It may be that NFB42 has a function different from other well-characterized F box-containing proteins that are involved in G1/S phase and G2/M phase events. To determine the potential functions of NFB42, attempts were made to generate stable neural cell lines expressing the protein; however, these initial attempts were unsuccessful. By following N1E-115 neuroblastoma cells during the period after transfection, it was clear why no stable transfected cells were obtained (Fig. 5). Cells were transfected with vector or NFB42 cDNA and selected with G418. Cell numbers decreased across both populations as cells lacking the resistance marker died; however, stably transfected cells in the vector-transfected populations began to proliferate, resulting in an eventual rise in cell number. In NFB42-transfected populations, no dividing cells were observed, indicating that the expression of the protein inhibited proliferation and/or survival.

The low basal proliferation rate of N1E-115 cells made it difficult to determine whether there were differences between vector- and NFB42-transfected cells soon after transfection; therefore, experiments were performed with CHO cells. These cells were transfected with vector, full-length NFB42, or deletion mutants lacking the PEST sequence (NFB42(Δ1–52)) or the PEST sequence and the F box domain (NFB42(Δ1–94)). These constructs were co-transfected with a GFP expression construct to identify transfected cells. Proliferation of GFP-positive cells was measured 18–66 h post-transfection. Vector-transfected cells proliferated after transfection, demonstrating an approximately 6-fold increase in cell number after 66 h (Fig. 6). This level of proliferation was similar to that observed (via phase-contrast microscopy) across the entire population. Expression of NFB42 or NFB42(Δ1–52) resulted in a dramatic inhibition of proliferation and an apparent growth arrest, because the cell number remained relatively constant over the time course analyzed. In contrast, expression of NFB42(Δ1–94) had no significant effect on proliferation, indicating that the F

while sequencing chromosome 3 of C. elegans; however, no function has been ascribed to it. Both NFB42 and C14B1.3 contain an F box motif (Fig. 1B). This domain is used to bind Skp1p, a protein required for entry into S phase and kinetochore function in budding yeast (17, 21). The amino-terminal region of NFB42 contains a PEST sequence rich in the amino acids proline, glutamic acid, serine, and threonine. PEST sequences are often found in proteins that have short half-lives and are rapidly degraded by the proteasome (31).

Northern blot analysis of different tissues indicates that NFB42 is a 1.4-kb mRNA expressed in the cerebral cortex, midbrain, and cerebellum/brainstem, but it is not present in the heart, lung, kidney, or skeletal muscle (Fig. 2). Even after overexposing Northern blots, mRNA is not detected in these non-neural tissues (data not shown), suggesting that NFB42 mRNA is highly enriched in the nervous system. Consistent with these results, Western blots indicate that NFB42 protein is present in all major areas of the brain but is not present in the non-neural tissues examined (Fig. 3). In these blots, NFB42 protein is expressed in the brain. Western blot of adult rat protein probed with rabbit anti-NFB42. Protein migrates at approximately 42 kDa. Similar to the skeletal muscle (SM) and lung, no expression was detected in the adult heart, kidney, liver, or spleen (data not shown). In these Western blots, approximately 25 μg of protein from each brain region were separated, compared with 75 μg for other tissues.

The presence of an F box in NFB42 suggests that it may be involved in cell cycle regulation; however, its localization to postmitotic neurons suggests that it is not involved in regulating typical cell cycle processes. It may be that NFB42 has a function different from other well-characterized F box-containing proteins that are involved in G1/S phase and G2/M phase events. To determine the potential functions of NFB42,
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FIG. 5. NFB42 expression inhibits N1E-115 cell growth. N1E-115 mouse neuroblastoma cells were transfected with NFB42 or vector alone. Two days post-transfection, the cells were split, and 400 µg/ml G418 was added to the culture medium. Cells were then counted for 3–15 days after the selection medium was added (shown as days 0–12). The relative cell number represents the ratio of the number of cells in randomly selected fields on a given day to the number of cells at time 0. The results shown are representative of five individual experiments.

FIG. 6. NFB42 expression inhibits CHO cell proliferation. CHO cells were co-transfected with a GFP expression construct and vector (●), NFB42 (■), NFB42(Δ1–52) (○), or p21(WAF1) (▲) DNA. 16 h after transfection, the cells were split, and GFP-positive cells in random fields were counted 2 h later (at 18 h). Cell counts at all subsequent time points were conducted in the same random fields selected at 18 h post-transfection. To determine the proliferation of the population, phase-contrast microscopy was used to obtain cell counts (population, △). Data represent the means ± S.E. from three individual experiments. In these experiments, the expression of transfected proteins was confirmed by Western blot analysis (data not shown).

DISCUSSION

In the budding yeast S. cerevisiae, Skp1p and F box-containing proteins play critical roles in cell cycle events, including G1/S phase transition and kinetochore function (17, 18–22). Although these are very different cellular processes, a common feature required in each is the degradation of positive or negative regulators. F box-containing proteins recognize targets to be degraded and link the targeted protein to the ubiquitin ligase complex through the ability of its F box to bind Skp1p (17, 18, 19). Currently, it is thought that proteins targeted for degradation are “marked” by phosphorylation, which allows for recognition by F box-containing proteins (19, 20). Phosphorylation of proteins followed by degradation is a feature of the ubiquitin-proteasome pathway (32, 33); however, it is not known if all proteins targeted for degradation after phosphorylation bind F box-containing proteins.

Whereas it is clear that Skp1p and F box proteins can control cell growth by targeting cell cycle regulators for destruction by the proteasome, an additional function of Skp1p is suggested by a recent study showing that p58, an F box protein required for kinetochore assembly, binds Skp1p and is then activated by phosphorylation. This interaction also targets p58 for destruction by the ubiquitin-proteasome pathway (22). Therefore, p58 is the first example of an F box protein that binds Skp1p for activation, rather than functioning to recognize a target protein and directing it to the proteasome pathway. The observation that an F box protein can be activated by binding to Skp1p and then be targeted for destruction may be relevant to NFB42, which contains an amino-terminal PEST sequence, suggesting that it is a short-
lived protein directed to the proteasome (31). Therefore, NFB42 may be the target of activation and destruction by the Skp1p pathway rather than part of a complex recognizing and targeting other proteins for degradation. Identifying proteins that bind NFB42 and determining whether NFB42 is phosphorylated (it has consensus phosphorylation sites for several kinases) should help define possible functional roles.

A better understanding of the role of NFB42 in Skp1p-dependent and independent events not only requires more information on NFB42 but also requires more information on the potential functions of Skp1p and F box proteins in higher eukaryotes. A possible link to growth control is suggested by studies showing that in transformed fibroblasts, cyclin A/cdk2 is present as a complex with p9, Skp1p, and Skp2p (an F box protein), rather than PCNA and p21^WAF1, which normally bind cyclin A/cdk2 in untransformed fibroblasts (23). Injecting Skp2p antibodies into both transformed and normal fibroblasts blocks entry into S phase, indicating that this F box protein is required for S phase entry in mammalian cells. Injecting antibodies to Skp1p does not alter cell cycle progression; however, the ability of these antibodies to inhibit Skp1p function could not be determined (23). Nevertheless, Skp2p, an F box-containing protein, is required for G1/S phase transition in normal and transformed fibroblasts, suggesting that mammalian cells have pathways similar to those in budding yeast that target cell cycle regulators for destruction by the proteasome. Obviously, characterizing these pathways and defining the additional functions of Skp1p-F box proteins in mammalian cells will be important for understanding the overall regulation of cell growth.

Based on data from _S. cerevisiae_ and limited information on cultured mammalian cells, a primary function of F box proteins is to help control various aspects of cell cycle/cell growth by targeting positive or negative regulators to the proteasome for degradation. The presence of NFB42 in postmitotic neurons, however, suggests that if F box proteins are involved only in regulating cell growth, then NFB42 must perform a growth-related function other than controlling typical cell cycle events. For example, NFB42 could help maintain neurons in a postmitotic state and/or regulate proteins in pathways that typically couple signal transduction to cell cycle control in proliferating cells. An example of the pathways present in both proliferating and postmitotic cells includes those involved in mitogen-activated protein kinase signaling. These proteins are known to control entry into the cell cycle (9) as well as diverse functions in postmitotic neurons, including the modulation of synaptic plasticity (10). It would seem critical for neurons that maintain proteins and signaling pathways that control proliferation in undifferentiated cells to have mechanisms protecting against attempts to enter the cell cycle. NFB42 may perform such a function in postmitotic neurons.

The restricted expression of NFB42 in neurons, along with its ability to grow both N1E-115 neuroblastoma and CHO cells, is consistent with NFB42 functioning to help maintain neurons in a postmitotic state and avoid an aberrant entry into the cell cycle. In CHO cells, the growth arrest appeared complete, with no evidence of any further proliferation. This inhibition of proliferation was dependent on the presence of the F box, because deletion of this domain abolished the growth inhibitory effects of NFB42. Obviously, the results from deletion analysis need to be interpreted cautiously; however, in the present study, a large deletion of the amino-terminal 52 amino acids (the PEST sequence) did not significantly alter function. Analysis of Skp1p interactions with NFB42 deletion mutants also demonstrates the importance of the F box domain, because the deletion of the F box inhibited interactions with Skp1p. At present, the mechanism by which NFB42 inhibits cell growth is unclear, although it is possible that the effects are due to sequestering Skp1p from normal cell cycle function. This also warrants further investigation, because this would represent the first report of Skp1p functioning in the mammalian cell cycle.

NFB42 is a novel protein with intriguing properties. It contains an F box that has been shown to be important in controlling various aspects of the cell cycle by targeting proteins for degradation by the proteasome. The F box of NFB42 appears to be required for binding Skp1p and inhibiting proliferation. Transfecting neuroblastoma or CHO cells induces growth arrest, which is consistent with a role in the control of the cell cycle; however, in the adult, NFB42 appears to be selectively expressed by postmitotic neurons. This restricted localization in a postmitotic cell suggests that NFB42 may be involved in other functions, such as helping neurons maintain a postmitotic state or enabling neurons to maintain some of the signal transduction pathways that activate cell cycle events in proliferative cells. NFB42 is not expressed in all neurons, although an analysis of database Expressed Sequence Tags indicate that there are apparent homologues present in the nervous system. Therefore, NFB42 likely represents the first of a family of proteins. Ongoing studies to identify proteins that interact with NFB42 and to further characterize the observed growth arrest should begin to define its function in postmitotic neurons and possibly in growth control and protein degradation by the ubiquitin-proteasome pathway.

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