Nucleomorphin

A NOVEL, ACIDIC, NUCLEAR CALMODULIN-BINDING PROTEIN FROM DICTYOSTELIUM THAT REGULATES NUCLEAR NUMBER*

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Probing of Dictyostelium discoideum cell extracts after SDS-PAGE using 35S-recombinant calmodulin (CaM) as a probe has revealed approximately three-dozen Ca2+-dependent calmodulin binding proteins. Here, we report the molecular cloning, expression, and subcellular localization of a gene encoding a novel calmodulin-binding protein (CaMBP); we have called nucleomorphin, from D. discoideum. A ZAP cDNA expression library of cells from multicellular development was screened using a recombinant calmodulin probe (35S-VU1-CaM). The open reading frame of 1119 nucleotides encodes a polypeptide of 340 amino acids with a calculated molecular mass of 38.7 kDa and is constitutively expressed throughout the Dictyostelium life cycle. Nucleomorphin contains a highly acidic glutamic/aspartic acid inverted repeat (DEED) with significant similarity to the conserved nucleoplasmin domain and a putative transmembrane domain in the carboxyl-terminal region. Southern blotting reveals that nucleomorphin exists as a single copy gene. Using gel overlay assays and CaM-agarose we show that bacterially expressed nucleomorphin binds to bovine CaM in a Ca2+-dependent manner. Amino-terminal fusion to the green fluorescence protein (GFP) showed that GFP-NumA localized to the nucleus as distinct arc-like patterns similar to heterochromatin regions. GFP-NumA lacking the acidic DEED repeat still showed arc-like accumulations at the nuclear periphery, but the number of nuclei in these cells was increased markedly compared with control cells. Cells expressing GFP-NumA lacking the transmembrane domain localized to the nuclear periphery but did not affect nuclear number or gross morphology. Nucleomorphin is the first nuclear CaMBP to be identified in Dictyostelium. Furthermore, these data present the first identification of a member of the nucleoplasmin family as a calmodulin-binding protein and suggest nucleomorphin has a role in nuclear structure in Dictyostelium.

Calmodulin (CaM1), the major, essential Ca2+-binding protein of all eukaryotes, is highly conserved such that the CaM of mammals and of eukaryotic microbes, such as Dictyostelium discoideum, differs in only a few amino acids leaving them functionally identical (1–4). CaM is a small acidic protein consisting of a flexible α-helical tether joining two globular domains each of which contain two Ca2+-binding sites (5, 6). Upon Ca2+ binding, CaM undergoes a large conformational change exposing two hydrophobic patches that allow for target-protein interaction (7). CaM binding to its targets does not operate through a conserved motif, because CaM binding regions on target proteins show little sequence homology. Many Ca2+-dependent CaMBPs have one or more CaM-binding domains characterized by a basic amphipathic helix commonly with positively charged residues interspersed among hydrophobic and aromatic residues (7). α-Helical wheel analysis typi- cally shows a segregation of hydrophobic residues on one side and basic charged residues on the other (8). Ca2+-dependent CaMBPs can be grouped into two related motifs (1-8-14 and 1-5-10) based on conserved hydrophobic residues (9–11). Proteins containing the 1-8-14 motif include calcineurin (CN), nitric-oxide synthase, adenylyl cyclase, and myosin light chain kinase, whereas those using the 1-5-10 motif include CaM kinase I (CaMKI), CaMKII, and synapsin (11–14). CaMBPs that do not recognize these motifs include adenylyl cyclase and dystrophin (15, 16). Many Ca2+-independent CaMBPs exist, adding to the complexity of CaM regulation and function. CaM also interacts in a Ca2+-independent manner through an IQ motif, as has been shown for conventional type II myosin light chains and the unconventional myosins, neuromodulin and neurogranin (17–19). Because CaM’s target proteins (CaMBPs) do the work, a full understanding of their structure, function, and regulation is essential, but, as yet, the full complement of CaMBPs has not been determined for any cell type.

Dictyostelium has long been used as a model organism for the study of the molecular biology of cell function and differentiation in eukaryotic cells (20). Dictyostelium is haploid and contains one CaM gene, and null mutants are lethal (1). Screening of D. discoideum cell extracts after SDS-PAGE using a recombinant CaM probe (35S-VU1-CaM) reveals <12 Ca2+-independent, ~3 dozen Ca2+-dependent CaMBPs plus a sole Ca2+-inhibited CaMBP (21–24). CaM and certain CaMBPs have been linked to specific events during starvation, asexual development, chemotaxis, gametogenesis, fertilization, and spore germination in D. discoideum (21–26). Despite their number and essential roles in a number of events, only a few CaMBPs have been characterized fully in Dictyostelium. Myosin heavy chain phosphatase; DIG, digoxigenin; GFP, green fluorescent protein; HRP, hors eradish peroxidase; LPS, lower pad solution; MBP, maltose binding protein; PVDF, polyvinylidene difluoride; CN, calcineurin; NLS, nuclear localization sequence; DEED, glutamic/aspartic acid inverted repeat; IPTG, isopropyl-β-D-thiogalactopyranoside; BSA, bovine serum albumin.

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The abbreviations used are: CaM, calmodulin; CaMBPs, calmodulin-binding proteins; CaMK, CaM kinase; CLAP, calf-intestinal alkaline phosphatase; DIG, digoxigenin; GFP, green fluorescent protein; HRP, horseradish peroxidase; LPS, lower pad solution; MBP, maltose binding protein; PVDF, polyvinylidene difluoride; CN, calcineurin; NLS, nuclear localization sequence; DEED, glutamic/aspartic acid inverted repeat; IPTG, isopropyl-β-D-thiogalactopyranoside; BSA, bovine serum albumin.
kinase, a Ca\(^{2+}\)/CaM-dependent enzyme involved in myosin assembly, has been shown to be integral in cell motility (27, 28). Myosin I isoforms are Ca\(^{2+}\)-independent CaM targets (17). Knockout mutants for α-actinin, a Ca\(^{2+}\)/CaM-dependent CaMBP that binds actin, show defects in motility and orientation (29). Knockout mutants for Dictyostelium IQGAP, a Ca\(^{2+}\)- independent CaMBP, are unable to complete cytokinesis (30).

Dictyostelium CN has been extensively characterized (31–33). FK506 and cyclosporin A do not inhibit growth or aggregation but do affect cell differentiation, yet the regulation and roles of Dictyostelium CN are still under analysis (34, 35). In addition to acting as CaM's effectors, CaMBPs bind different targets based upon cytosolic Ca\(^{2+}\) levels; they localize CaMs to subcellular locales and act as capacitors in CaM function (36). Until all of the CaMBPs have been revealed and their functions elucidated, any model of cellular processes involving Ca\(^{2+}\) and CaM will remain incomplete.

Our approach has been to screen a AZAP cDNA expression library of cAMP-chemoresponsive cells from multicellular development using a recombinant CaM probe (35S-VU1-CaM) to isolate cDNAs encoding putative CaMBPs. This approach was validated when the first cDNA that we isolated and sequenced encoded calcineurin A (GenBankTM accession number U22397), the sole Ca\(^{2+}\)/CaM-dependent protein phosphatase of eukaryotes. Similar approaches have been used to identify novel CaMBPs in plants, including maize pollen calmodulin-binding channel protein (37, 38). Here we report on the isolation of a novel nuclear Dictyostelium calmodulin-binding protein, and a tobacco plasma membrane calmodulin-binding channel protein (37, 38). Here we report on the isolation of a 1119-bp cDNA encoding a novel, acidic, nuclear protein of 340 amino acids with a predicted molecular mass of 38.7 kDa that contains several nuclear localization sequences (NLS) plus an extensive, continuous 52-amino acid inverted repeat of glutamic and aspartic acid residues (DEED repeat). This region is characteristic of the conserved nucleoplasmin domain suggesting it is a member of the nucleoplasmin superfamily of nuclear proteins. We have named this gene numA for nucleoplasmin.

Southern blotting and PCR indicate that a single gene exists in the genome of D. discoideum. Binding assays using truncated fusion proteins show nucleoplasmin to be a Ca\(^{2+}\)-dependent CaM target protein that contains at least two CaM-binding domains. Nucleoplasmin mRNA and protein are each expressed continuously throughout development. GFP-NumA localized specifically to several arc-like, heterochromatic regions along the periphery of the nucleus. GFP-NumA lacking the putative transmembrane domain and the DEED repeat still accumulated at the periphery of the nucleus; however, removal of the acidic region leads to an increase in multinuclearity. Together these data indicate that nucleoplasmin is a nuclear calmodulin-binding protein and is related to nucleoplasmin, which may be involved in nuclear structure in Dictyostelium.

**EXPERIMENTAL PROCEDURES**

**Materials**—All restriction enzymes used were purchased from Amersham Biosciences, Inc. T4 DNA ligase and T4 polynucleotide kinase were purchased from New England BioLabs. All chemicals, including media reagents, were purchased from BioShop Canada, VWR Scientific Products, or Sigma-Aldrich. TRIZol RNA isolation reagent and low DNA mass ladders were purchased from Invitrogen. Expand High Fidelity PCR kit, High Pure PCR Template purification kit, DIG DNA Labeling and Detection kit, DIG Easy-Hyb, nylon membranes, PCR molecular weight markers, and DIG-labeled DNA and RNA molecular weight markers were from Roche Molecular Biochemicals. PCR primers were purchased from Sigma-Genosys. Qiagen II Gel Extraction system was purchased from Qiagen. The pET21-b(+)- vector was obtained from Novagen.

**Screening of the A ZAP cDNA Expression Library Using 35S-VU1-CaM**—Titration of bacteriophage and screening of the cDNA library were essentially carried out as described previously (39). Variations in protocol were implemented with respect to the use of \(^{35}\)S-radiolabeled recombinant CaM as a probe. An overnight culture of an XL1-Blue strain of Escherichia coli in LB media containing 0.2% maltose was pelleted and resuspended in 10 mM MgSO\(_4\). Serial dilutions of bacteriophage ranging from 10^{-4} to 10^{-10} were prepared in SM buffer (100 mM Tris-HCl, pH 7.5, 50 mM MgSO\(_4\), 0.5 mM EDTA, and 0.5% sucrose) (39). Diluted phage was mixed 1:1 with XL1-Blue E. coli and incubated at 37 °C. Three milliliters of liquid LB agarose was added and spread evenly over a 90-mm LB agar plate then incubated at 42 °C for 2 h. Nitrocultelllose membranes were soaked in 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG), dried, and placed over the surface of the agar plate; plates were incubated at 30 °C overnight. Membranes were removed from the plates, dried, and then washed in CaM-probing buffer (50 mM Tris-HCl, pH 7.0, 200 mM KCl, 1 mM CaCl\(_2\), and 0.05% Tween 20). Membranes were blocked for 2–3 h in CaM-probing buffer with 3% BSA and 0.05% sodium azide added, then probed at room temperature for 3 h with 3 ml of recombinant \(^{35}\)S-VU1-CaM (3.6 × 10^{6} to 9.0 × 10^{6} cpm/ml) dissolved in CaM-probing buffer supplemented with 3% BSA and 0.05% sodium azide. The production of \(^{35}\)S-VU1-CaM is detailed elsewhere (40). The membranes were then washed with CaM-probing buffer four consecutive times for a period of 10 min each. Membranes were dried and exposed to autoradiography film (Kodak X-Omat AR Scientific Imaging film) for 1–2 days. Single plaques corresponding to positive signals on the autoradiography film were located and subjected to three freeze–thaw cycles before being referred to as cDNA inserts coding for putative calcium-dependent calmodulin-binding proteins. Excision of the pBluescript phagemid and infection of host bacterial cells was conducted according to the protocol provided with the ExAssist Intermediate-Resistant Helper Phage kit purchased from Stratagene. Plasmids were isolated from positive clones by way of alkali lysis (39) and subjected to restriction analysis using Styl, HindIII, BstEII, and PstI. Clones with dissimilar restriction enzyme fragments were sequenced at the Core Molecular Biology Facility, York University. Sequences were compared with those in the GenBankTM Data base using BLAST (41). Protein motifs were identified, and the predicted molecular weight and isoelectric point were determined from the deduced amino acid sequences using the programs PSORT, version 6.4 (42), Prosite, and the ExPaSy Molecular Biology Server (Swiss Institute of Bioinformatics).

**Cell Lines and Cultures**—D. discoideum AX2 was used as the wild type strain. Cells were cultured axenically in HL-5 at 22 °C by guest on July 23, 2018http://www.jbc.org/ Downloaded from

**Construction of pMAL and pTX-GFP Expression Vectors**—PCR primers were designed to amplify the entire nucleoplasmin open reading frame coding for amino acids 1 through 340. EcoRI and HindIII sites were added to the 5' and 3'-ends, respectively, using the primers 38F-ER1 (5'-TGAACTCGATGGTGCATTAAAAATACAT-3') and 38R-H3 (5'-TAATTGAACCTTTATTTGAGGCTA-3'). Using the Expand High Fidelity PCR kit from Roche Molecular Biochemicals,
PCR was performed in a GeneAmp PCR System 9600 (PerkinElmer Life Sciences) for 25 cycles (each consisting of a denaturation at 94 °C for 2 min, annealing at 50 °C for 2 min, and extension at 72 °C for 1 min). A single 1035-bp fragment was amplified. Prior to sub-cloning into pUC19, which had previously been digested with SmaI, the ends were religated using T4 polynucleotide ligase and transformed into E. coli strain DH5α. The recombinant plasmid was purified and was designated pUC38. Digestion of pUC38 with EcoRI and HindIII liberated the 1035 bp fragment, which was agarose gel-purified using the Qiagen QIAEX II gel purification kit and ligated into pMal-C2 digested with EcoRI and HindIII, and cloned into E. coli strain TB1. The recombinant plasmid was purified, sequenced, and designated pMAL-NumA.

**NumA9126—**PCR primers were designed to amplify the region encoding amino acids 1 through 1034. EcoRI and HindIII sites were added to the 5’- and 3’-ends, respectively, using the primers 38F-EcoRI and 38-bpC-HindIII (5’-GAAGCTTATTTAATCATCATATTTATAC-3’). PCR was performed as outlined above generating a single 926-bp fragment that was sub-cloned into pUC19 and pMAL as described, sequenced, and named pMAL-NumA9126.

**NumA218—**PCR primers were designed to amplify the region encoding amino acids 1 through 1022. EcoRI and HindIII sites were added to the 5’- and 3’-ends, respectively, using the primers 38F-EcoRI and 38-bpE-HindIII (5’-GAAGCTTATTTAATCATCATATTTATAC-3’). PCR was performed as outlined above generating a single 951-bp fragment that was sub-cloned into pUC19 and pMAL as described, sequenced, and named pMAL-NumA218.

**NumA218N—**PCR primers were designed to amplify the region encoding amino acids 1 through 1018. EcoRI and HindIII sites were added to the 5’- and 3’-ends, respectively, using the primers 38F-EcoRI and 38-bpE-HindIII (5’-GAAGCTTATTTAATCATCATATTTATAC-3’). PCR was performed as outlined above generating a single 905-bp fragment that was sub-cloned into pUC19 and pMAL as described, sequenced, and named pMAL-NumA218N.

**GFP-NumA—**PCR primers were designed to amplify the entire nucleomorphin open reading frame coding for amino acids 1 through 340. To facilitate cloning into pTX-GFP, a SacI site was added to the forward primer 38F-SacI (5’-GAAGCTTCTATTAATCATCATATTTATAC-3’), and an XhoI site (5’-ACTCGAGTATTTGAGTGATG-3’) to the reverse primer. PCR was performed and generated a single 1043-bp fragment that was sub-cloned into pUC19 and pTX-GFP as described, sequenced, and named pGFP-NumA. The 905-bp fragment was cloned into pTX-GFP as described, sequenced, and named pGFP-NumA118-167.

**GFP-NumA118—**Removal of residues 118–167 was performed as described below. PCR was performed as outlined for pGFP-NumA. The 905-bp fragment was cloned into pTX-GFP as described, sequenced, and named pGFP-NumA118-167.

**GFP-NumA167—**Removal of residues 305–340 is described above. PCR was performed as outlined for pGFP-NumA with the exception of the reverse primer. This primer incorporates an XhoI site to (5’-ACTCGAGTATTTGAGTGATG-3’) to the reverse primer. PCR was performed and generated a 380-bp fragment that contained a restriction site for NcoI. A single 905-bp fragment was amplified, subcloned into pUC19 and pET21b(+), and named pETNumA118-167.

**Protein Purification and Production of Antibodies—**E. coli strain TB1 expressed only pMAL-NumA118N and pMAL-NumA118C218 to any detectable levels, and both were purified by malto-oligosaccharide chromatography. Each fraction was analyzed by SDS-PAGE and Coomassie brilliant blue staining. Purified protein was eluted by the third day of selection. Single colonies were aspirated using sterile Pasteur pipettes into 2 ml of HL-5 containing 10 μg/ml G418 and grown axenically to saturation as described above.

**Imaging of GFP-NumA Clones—**To record the distribution of GFP-NumA in living cells, cells were grown to a density of 5 × 10⁶ cells/ml,
washed in low ionic strength DB buffer, resuspended at a density of 1 × 10^6 cells/ml, and applied to cover slips. Cells were allowed to adhere to the cover slips for a period of 1 h, which also served as a period of starvation to allow cells to digest endocytosed nutrient medium that is available. To observe nuclei, cells were stained with Hoechst 33258. Phase contrast and fluorescent images were taken using a Nikon Optiphot-2 epifluorescence microscope with a 100× Neofluor oil immersion objective and a differential interference contrast filter. For exciting GFF, the microscope was equipped with a UV excitation immunofluorescence fluorescein isothiocyanate filter set with a dichroic mirror (DM-400, Nikon) and barrier filter (BA-435, Nikon). Images were captured using a Nikon FX-35DX camera attached to a Microflex electronic shutter (UXF-DX, Nikon). At least 400 cells from each experiment were examined, and the number of nuclei per cell was counted.

RESULTS

Isolation of a Novel Dictyostelium CaM-binding Protein, Nucleomorphin—We have used a protein-protein interaction screening system to isolate cDNAs encoding putative Ca^2+–dependent CaM-binding proteins from CAMP-chemoresponsive cells of D. discoideum. A AZAP cDNA expression library constructed from cells 10–16 h through development was screened using recombinant 35S-VU1-CaM. Single plaques corresponding to positive signals on autoradiographic film were subjected to three successive screens in which the amount of 35S-VU1-CaM was reduced by a factor of 10, respectively (data not shown). After the high stringency screening, positive signals were deemed putative Ca^2+–dependent CaMBPs. Of those recombinants detected, two clones were isolated that coded for nucleomorphin, all of which bound CaM in a Ca^2+–dependent manner. Restriction enzyme analysis of each cDNA revealed identical banding patterns for each, suggesting they were derived from the same clone. Sequencing of the two cDNAs revealed that they were identical.

Sequence and Structural Features of Dictyostelium Nucleomorphin—Nucleomorphin is encoded by a cDNA of 1119 bp. The nucleotide sequence and the deduced amino acid sequence of this gene are shown in Fig. 1. The cDNA contains a 49-bp extension at the 5′-end, and the sequence upstream of the ATG start codon (GTAAATGG) differs slightly from a consensus translation initiation sequence (AAAAATG) of D. discoideum (46). The termination codon (UAA) occurs at 1070 bp. The AT-rich Dictyostelium genome has resulted in an extreme codon bias and thus Dictyostelium genes almost exclusively use UAA as their termination codon (47, 48). Translation of the sequence predicts a single open reading frame encoding a protein of 340 amino acids with a calculated molecular mass of 38.7 kDa and an isoelectric point of 4.83. Fig. 1 shows the amino acid sequence of nucleomorphin and highlights interesting domains contained within. One feature is the somewhat long stretches containing serine (amino acids 6–16 and 295–306). A stretch of serine, threonine, and asparagine is commonly seen in many Dictyostelium proteins but is of unknown function (49). Blast analysis of nucleomorphin did not reveal any significant matches to known protein homologs. Within nucleomorphin resides an extensive, highly acidic domain (amino acids 121–172) that is made up of 35% aspartic acid and 54% glutamic acid residues, which we call the DEED repeat. This region has an interesting inverted repeat of which the significance, if any, remains unclear. A PSI- and PHI-BLAST search of this domain produced significant similarities to Pfam nucleoplasm proteins from a variety of species (including mitotic mutants) proteins (PF2 from Schizosaccharomyces pombe) (50), a nucleoplasm-like protein (CRP-1) from Drosophila (51), and nucleoplasm from chicken (52). An alignment of the conserved domain using ClustalW shows the presence of high percentage similarities (Fig. 2). Based on further analysis with PSORT, version 6.4 (42), nucleomorphin contains a putative transmembrane region (residues 319–335) typical of an Nt (N tail) membrane protein, because it does not contain a detectable cleavage signal sequence and the region exists in the carboxyl terminus (53). Discrimination of nuclear localization signals (NLS) reveals the presence of four possible regions. At position 61 in the amino acid sequence exists a classical NLS (residues RPRK) with two more found at positions 31 and 246 (residues PKKKKKK and PTKKKRL), respectively. The fourth NLS is consistent with that of the bipartite classification, found at position 48 in the amino acid sequence (residues KKKYQPDEIIAHSRPRK). There are also four conserved dileucine residues within nucleomorphin positioned at residues 23, 44, 181, and 234. If nucleomorphin undergoes post-translational modifications at any of the potential sites listed in Fig. 1, it may explain why this protein whose predicted molecular mass of 38.7 kDa migrates on SDS-PAGE gels with an apparent molecular mass of 43 kDa. Although, one cannot rule out the possibility that the highly acidic nature of nucleomorphin results in anomalous migration rates through SDS-PAGE due to its poor ability to bind SDS. This protein is known to demarcate the dictyosome and appears to be involved in anclor centerome protein B cDNAs (54). The protein responsible for high stringency CaM binding, nucleomorphin (81–94 and 172–194), are presented in Fig. 3. Within the amino terminus of nucleomorphin is a predicted CaM-binding domain (residues 81–94) of the 1–14 motif; the carboxyl terminus contains two more potential domains, a 1–14 motif is present (residues 182–194) and a putative domain that contains characteristics of the 1–10 motif (residues 172–185).
Each construct was used to begin mapping the location of the CaM-binding domain. Overlay analysis with 35S-CaM showed that each fusion protein does not bind CaM, thus binding must reside within the DEED repeat of those constructs containing the DEED repeat. The maltose-binding protein domain sequences. Those containing the DEED repeat (pMAL-NumA
\textsubscript{118–167} and pMAL-NumA
\textsubscript{C218}) and those that lacked the DEED repeat (pMAL-NumA
\textsubscript{C36} and pMAL-NumA
\textsubscript{C160}) and those containing the DEED repeat (pMAL-NumA
\textsubscript{118–167}) were grown with or without IPTG as described above.

The positions of alignment: nucleomorphin (slime mold) AF149042; Mitotic apparatus protein p62 (sea urchin) P91753; nucleomorphin-like protein NOVA (African clawed frog) O42554; nucleolasmorphin CAA29460; nucleophosmin (chicken) F16039. Residues that are identical in all four sequences are shaded in black. Homologous residues are shaded in gray.

\textbf{Nucleomorphin Is a Ca\textsuperscript{2+}-dependent Calmodulin-binding Protein.}—The full-length cDNA was cloned in-frame into both prokaryotic expression vectors pMALc2 and pET21-b(+). Attempts at overexpression of nucleomorphin as a fusion protein for purification purposes using either system was unsuccessful. The fusion protein from IPTG-induced cell cultures could not be detected by Coomassie Blue staining of SDS-PAGE gels and was only detected using serum antibodies against MBP or T7 tag (data not shown). We hypothesized that the 52-amino acid stretch (residues 121–172) of repeating Asp/Glu was affecting the translation of nucleomorphin in \textit{E. coli}. To test this, we constructed a number of truncated MBP-nucleomorphin fusion proteins: those containing the DEED repeat (pMAL-NumA
\textsubscript{118–167} and pMAL-NumA
\textsubscript{C218}) and those that lacked the DEED repeat (pMAL-NumA
\textsubscript{C36} and pMAL-NumA
\textsubscript{C160}) and those containing the DEED repeat (pMAL-NumA
\textsubscript{118–167}) was detected and found to be expressed constitutively throughout development.

\textbf{Expression and Immunodetection of Nucleomorphin during Development.—}Nucleomorphin expression was assessed by Northern blots of total RNA isolated from cells at 4-h intervals during filter development. A single transcript of about 1.4 kb was detected and found to be expressed constitutively throughout development (Fig. 6A). To determine the native size of the protein, total protein was also extracted at 4-h intervals during filter development, separated by SDS-PAGE, and blotted onto PVDF membranes, and nucleomorphin was detected using affinity-purified nucleomorphin antibody. As shown in Fig. 6B, nucleomorphin migrates with an apparent molecular mass of 43 kDa in SDS-PAGE and is present throughout development.
Fig. 5. The putative CaM-binding domains of nucleomorphin analyzed by helical wheel and CaM-binding assays. A, helical wheel representation of the putative CaM-binding sequences in nucleomorphin shows the segregation of hydrophobic and positively charged residues. The amino terminus domain includes residues 79–96; carboxyl terminus domains include residues 173–190 and 180–197, respectively. B, binding of 35S-radiolabeled calmodulin to pMAL-NumAΔC218 and pMAL-NumAΔN165. Lanes 1 and 2 represent 100 and 200 ng of purified pMAL-NumAΔC218; lanes 3 and 4 are loaded with 100 and 200 ng of purified pMAL-NumAΔN165. After fixation, gels were incubated overnight at 4 °C with 1 μCi/ml 35S-VU1-Calmodulin dissolved in probe buffer (200 mM KCl, 0.1% BSA, and 1 mM CaCl2). For Ca2+-independent assays, 2 mM EGTA was substituted for the 1 mM CaCl2 in the probe buffer. After washing and fixing, bound CaM was detected through exposure to autoradiography film. C, CaM-agarose pull-down assay of pETNumAΔ118–167. The assay was performed as described under “Experimental Procedures.” Insoluble extracts from cells expressing pETNumAΔ118–167 were incubated with CaM-agarose, washed free of non-bound protein, eluted by boiling in sample buffer, and resolved using 10% SDS-PAGE. Gels were stained with Coomassie Blue, destained, and dried. Lane 1, insoluble protein fraction; lane 2, eluted protein from CaM-agarose. Molecular mass of the fusion proteins in kilodaltons is shown.

The protein levels slightly increase during the first 4 h and then begin to decrease through development (Fig. 6B). These results may indicate an essential role for nucleomorphin in the life cycle of Dictyostelium. Genomic Southern blot analysis was performed using the DIG-labeled cDNA probe after digestion of genomic DNA with the restriction enzymes AscI, BglII, HindIII, and EcoRI (Fig. 7). The BglII digest resulted in the detection of two hybridizing bands as expected, because a single site for this restriction enzyme occurs in the middle of the cDNA and only one band was detected in the remaining lanes. This indicates that a single gene encoding nucleomorphin resides in the genome of D. discoideum. The PCR was used with primers designed from the cDNA to amplify the entire open reading frame using genomic DNA as the template. A single product was amplified identical in size to the cloned cDNA suggesting the gene for nucleomorphin is uninterrupted by introns (data not shown). DNA sequencing confirmed this and also verified the integrity of the cDNA encoding nucleomorphin.

Subcellular Localization of GFP-NumA in Vegetative Amoeba—We have used a GFP tag to study the localization of nucleomorphin in vivo. Because the carboxyl terminus contains a predicted single-pass transmembrane domain that may provide structural elements necessary for membrane associations, we decided to fuse GFP to the amino-terminal end of nucleomorphin. The intensity of GFP-NumA fluorescence in all of our transfectants was moderate compared with cells transfected with GFP alone (Fig. 8A). To ensure that the observed fluorescence is due to the intact fusion protein, Western blots were performed on total protein extracts using a monoclonal anti-GFP antibody (Sigma). A band was detected with a molecular mass of ~70 kDa corresponding to the predicted mass of the fusion protein (data not shown). However, a band of ~31 kDa was also observed representing the GFP, which may explain the presence of slight background intracellular fluorescence. Additionally, we also observed that fluorescence levels varied broadly from cell to cell, a common problem related to the actin15-promoter used to drive expression of the GFP fusion protein (55, 56). In vegetative cells, GFP-NumA was almost exclusively within the nucleus appearing as distinct arc-like bands that corresponded to heterochromatin-like domains adjacent to the nuclear membrane (57) (Fig. 8B). Cells were treated with Hoechst 33258, a specific stain for AT-rich regions of double-stranded DNA to verify the nuclear localization of the GFP constructs. Fig. 8C shows GFP-NumA is localized within nuclei stained with Hoechst.
Hoechst 33258 and GFP-NumA fluorescence computer-assisted image panel. GFP-NumA fluorescence. Right panel seen to occur at distinct locations at the periphery of the nucleus. Left echst 33258. Heterochromatin-like domains. Right panel lecular weight size markers are indicated on the Membranes were hybridized and washed under high stringency. Mo-

RI (Bgl3) or NumA. Left panel show a uniform distribution of GFP. Slips for a period of 1 h. Actinum cells were washed in DB buffer and allowed to adhere to cover.

FIG. 7. Southern blot analysis of genomic DNA from D. discoi-
deum strain AX3. DNA (1 µg) was digested with AseI (1), HindIII (2), EcoRI (3), or BglII (4) size fractionated on a 0.7% agarose gel, transferred to nylon membranes, and probed with DIG-labeled 0.9-kb cDNA. Membranes were hybridized and washed under high stringency. Molecular weight size markers are indicated on the left.

FIG. 8. Localization of GFP-NumA in vegetative cells. Dictyos-
estium cells were washed in DB buffer and allowed to adhere to cover slips for a period of 1 h. A, control cells transfected with pTX-GFP. Cells show a uniform distribution of GFP. B, cells transfected with GFP-NumA. Left panel, phase image showing dark patches corresponding to heterochromatin-like domains. Right panel, nuclear staining with Hoechst 33258. C, distribution of GFP-NumA in live vegetative cells is seen to occur at distinct locations at the periphery of the nucleus. Left panel, GFP-NumA fluorescence. Right panel, nuclear staining with Hoechst 33258 and GFP-NumA fluorescence computer-assisted image overlay.

The Effects of Expression of GFP-NumA Constructs Lacking the Tm or DEED Repeat—Cells expressing GFP-NumAΔC36 lacking the putative transmembrane domain or the acidic DEED repeat also retain their localization to heterochromatin-like domains at the periphery of Hoechst-stained nuclei (Fig. 9). However, those cells expressing the GFP-NumAΔ118–167 construct consistently displayed a dramatic increase in multinuclearity with as many as 16 nuclei in one cell (Fig. 9A). In contrast, wild type AX3 cells and the other GFP-NumA cell lines typically had one or two nuclei (Figs. 8 and 9C). It must be noted that pTX-GFP is an extrachromosomal vector, and integration into the genome does not occur. Therefore, the phenotypes observed are a result of overexpression of GFP-NumAΔ118–167 in the presence of the wild type nucleomorphin. The number of nuclei per cell was counted for each GFP transformant (Fig. 10). Cells expressing GFP-NumAΔ118–167 were rarely mononucleate compared with GFP-NumA and GFP-NumAΔC36 transformants with 28.9% of the cells having four or more nuclei. None of the constructs seemed to alter the size of the nuclei except in the case of cells with extremely high numbers of nuclei. To this end, we are beginning detailed analyses of the nuclear and cytoplasmic volumes during growth and development.

DISCUSSION

To understand the roles of calmodulin, the major eukaryotic Ca2+ sensor protein in Dictyostelium, we screened a λ Zap cDNA expression library with 35S-radiolabeled CaM to isolate cDNAs encoding some of its target proteins. Single plaques corresponding to positive signals identified on autoradiography film were selected resulting in the isolation of a cDNA encoding a novel CaM-binding protein. Using different approaches we have shown that the protein product, which we have called nucleomorphin, encoded by the cDNA binds to CaM in a Ca2+-dependent manner. First, the cDNA was identified through plaque screening where binding to CaM was detected in the presence of Ca2+, but not EGTA (data not shown). Second, in experiments using truncated forms of nucleomorphin fused to MBP, nucleomorphin bound to 35S-CaM in a Ca2+-dependent manner using an SDS-gel blot overlay assay (Fig. 5). The CaM binding intensities observed on the gel overlay are very similar suggesting CaM binds each fusion with comparable affinity. Proteins, including dystrophin have been shown to contain multiple CaM binding sequences found in both the amino and carboxyl termini (16). Specifically, we have looked for amino acid sequences, ~20–24 residues in length, that match the described structural features of known CaM-binding proteins, including helical wheel analysis, conserved hydrophobic residues, propensity to form an α-helix, and net charge (8, 11). We have identified three potential CaM-binding domains in nucleomorphin based upon these criteria (Fig. 3). These regions of sequence show characteristics typical of other calmodulin-binding sequences in that they are predominantly positively charged and contain an abundance of hydrophobic residues with aromatic residues interspersed. The calmodulin-binding sequences of CaMKI, CaMKII, utrophin, caldesmon, smooth muscle myosin light chain kinase, myristoylated alanine-rich c kinase substrate protein, synapsin, and myosin all show these cationic-hydrophobic chemical characteristics (3, 7). Within the amino terminus of nucleomorphin is a predicted CaM-binding domain (residues 81–94) of the 1–14 motif that was initially described (11). The main feature of this domain is the spacing of 12 amino acids between two bulky hydrophobic residues. Some CaMBPs of this type also contain conserved hydrophobic residues at positions 8 and/or 5 within the spacer region, although it is still unclear at this time whether the presence of these residues affects the binding affinities of CaM. Within the carboxyl terminus of nucleomorphin, a second potential 1–14 motif is present (residues 182–194). A third putative CaM-binding domain can be found in nucleomorphin (residues 172–185) that contains characteristics of the 1–10 motif, in which two bulky hydrophobic residues are spaced by eight amino acids. Many, but not all, known CaMBPs with this domain also have a conserved bulky hydrophobic residue positioned at

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amino acid 5 (11). The Ca\(^{2+}\)-dependent binding of nucleomorphin was further confirmed using bacterial expressed protein on CaM-agarose (Fig. 5). This assay used the pETNumA\(_{118-167}\) construct lacking the DEED repeat (residues 121–172) but retained the predicted CaM-binding domains. We hypothesized that this long stretch of acidic residues was affecting the translation of nucleomorphin in \textit{E. coli} by putting high demands on the need for each charged tRNA, respectively. This would likely result in pausing of the ribosome complex, halting translation, and leading to instability in the transcript. Expression of these constructs containing the Asp/Glu repeat could only be detected using serum antibodies against MBP or the T7 tag (data not shown). The concentration of CaM used in the screening of the library and subsequent CaM-binding series of experiments falls within the range of physiological CaM concentrations in the cell supporting evidence that nucleomorphin plays a physiological role in \textit{Dictyostelium}. To this end, we are beginning site-directed mutagenesis experiments to identify the exact sequences responsible for CaM binding.

Data base searches failed to reveal a homolog of nucleomorphin. However, the sequence contains a putative conserved domain of the superfamily of proteins known as nucleoplasmins. Other evidence also suggests nucleomorphin is a nuclear protein. There are four predicted NLS within the amino acid sequence of nucleomorphin. Of these, one NLS is consistent with that of the bipartite classification, found at position 48 in the amino acid sequence (residues KKSQDPIIAHSRPRK), which is consistent with nucleoplasmin (58). The significance of the NLS in nucleomorphin has yet to be shown, but experiments to delete these regions by site-directed mutagenesis will likely resolve this question. In addition to this is the presence of multiple potential phosphorylation sites for such kinases as casein kinase II, protein kinase A, and protein kinase C. Multiple phosphorylation sites and nuclear localization signals are also characteristics of nucleoplasmin and nucleophosmin (59, 60). Nucleomorphin is a highly acidic protein largely in part to the 52-amino acid region of Asp/Glu residues. Amino acid comparison of nucleomorphin with members of the nucleoplasmin family reveals sequence identities and similarities confined to the acidic domain. All members of this family have one or more acidic domains consisting of a total of 17 to more than 100 glutamic acid and aspartic acid residues per molecule. Sequence analysis shows that these two residues can comprise more than 25% of the total amino acids in some proteins of this superfamily; in nucleomorphin, they make up \(\sim 21\%\) of the total residues. Other chromatin-associated proteins, such as p62 (50), also contain acidic domains. Although nucleomorphin does not contain an identifiable DNA-binding domain, members of the nucleoplasmin family, including nucleoplasmin 3 and nucleoplasmin also lack such domains (61). The DEED repeat may also explain the anomalous molecular mass in SDS-PAGE. Like p62, CRP-1 from \textit{Drosophila} and \textit{Xenopus} nucleoplasmin, the predicted molecular mass of nucleomorphin (38 kDa) is less than the apparent mass calculated from SDS-PAGE mobility (43 kDa). The larger apparent mass may be due to the acidic properties of the protein as was shown for p62 (50). Although phosphorylation can contribute to the increase in mass, the phosphorylation state of nucleomorphin is still unknown. In
keeping with the acidic properties of nucleomorphin, the classes of proteins called A-proteins have also been shown to contain extensive stretches of acidic residues (50). These proteins are a complex group that vary in both structure and function, yet demonstrate a significant binding to the core histones in vivo (62, 63). This suggests that the acidic domain within nucleomorphin could serve to bind to core histones or other positively charged chromatins-associated proteins. It has been shown that nucleoplasm in binding to the core histones mediates chromatid decondensation, nucleosome formation, and DNA transcription and is believed to be essential in assembling nucleosomal arrays, although precise functions and the mode of action remain to be elucidated (58, 63–65).

In Dictyostelium, nucleomorphin mRNA and protein are maintained throughout development at relatively steady levels. Expression of GFP-NumA was analyzed by Western blotting using monoclonal anti-GFP antibodies, and it was found that the construct, although present in appreciable amounts, was being degraded. Furthermore, it accounts for the appearance of weak GFP fluorescence throughout the cell. This suggests nucleomorphin expression is tightly regulated so as to decrease in nuclear number in constructs lacking the DEED repeat strongly suggest that this protein is involved in some fundamental organization of the Dictyostelium nucleus.

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