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Nucleolin Is a Calcium-Binding Protein

James S.C. Gilchrist,[1]* Bernard Abrenica,[1] Patrick J. DiMario,[2] Michael P. Czubryt,[3] and Grant N. Pierce[3]

[1]Department of Oral Biology and Physiology, Division of Stroke and Vascular Disease, University of Manitoba, Winnipeg, Manitoba
[2]Department of Biological Sciences, Louisiana State University, Baton Rouge, Louisiana
[3]Department of Physiology, Division of Stroke and Vascular Disease, University of Manitoba, Winnipeg, Manitoba

Abstract We have purified a prominent 110-kDa protein (p110) from 1.6 M NaCl extracts of rat liver nuclei that appears to bind Ca^{2+}. p110 was originally identified by prominent blue staining with 'Stains-All' in sodium dodecyl sulfate–polyacrylamide gels and was observed to specifically bind ruthenium red and $^{45}Ca^{2+}$ in nitrocellulose blot overlays. In spin-dialysis studies, purified p110 saturably bound approximately 75 nmol Ca^{2+}/mg protein at a concentration of 1 mM total Ca^{2+} with half-maximal binding observed at 105 μM Ca^{2+}. With purification, p110 became increasingly susceptible to proteolytic (likely autolytic) fragmentation, although most intermediary peptides between 40 and 90 kDa retained ''Stains-All'', ruthenium red, and $^{45}Ca^{2+}$ binding. N-terminal sequencing of intact p110 and a 70-kDa autolytic peptide fragment revealed a strong homology to nucleolin. Two-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)/IEF revealed autolysis produced increasingly acidic peptide fragments ranging in apparent pI's from 5.5 for intact p110 to 3.5 for a 40 kDa peptide fragment. Intact p110 and several peptide fragments were immunostained with a highly specific anti-nucleolin antibody, R2D2, thus confirming the identity of this protein with nucleolin. These annexin-like Ca^{2+}-binding characteristics of nucleolin are likely contributed by its highly acidic argyrophilic N-terminus with autolysis apparently resulting in largely selective removal of its basic C-terminal domain. Although the Ca^{2+}-dependent functions of nucleolin are unknown, we discuss the possibility that like the structurally analogous HMG-1, its Ca^{2+}-dependent actions may regulate chromatin structure, possibly during apoptosis. J. Cell. Biochem. 85: 268–278, 2002.

Key words: nucleus; nuclear matrix; liver; Stains-All; electrophoresis; calsequestrin; protein purification

It is now clear that intracellular Ca^{2+} is an important modulator of nuclear gene expression [Carafoli et al., 2001; Mellstrom and Naranjo, 2001]. From an earlier appreciation of its central role during epigenesis [Whitaker and Patel, 1990], subsequent studies have revealed complex spatio–temporal Ca^{2+}-dependent regulation of gene transcription [Ghosh and Greenberg, 1995; Li et al., 1998]. Evidence suggests cytosolic Ca^{2+} waves, encoded as a frequency-modulated signal [Li et al., 1998], may propagate directly into the nucleus across the nuclear envelope or activate Ca^{2+}-dependent control points in second messenger pathways that ultimately converge upon gene transcriptional regulators in the nucleus.

Within the last few years, significant progress has been made in identifying important nuclear Ca^{2+}-dependent targets involved in gene regulation. These include: (a) calmodulin and S-100 proteins known to bind basic

Abbreviations used: BCIP, 5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine salt; CHAPS, 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propane sulfonate; DNA, deoxyribonucleic acid; DTT, dithiothreitol; ER, endoplasmic reticulum; IEF, isoelectrofocusing; IgG, immunoglobulin; kDa, kilodalton; NBT; *p*-nitro blue tetrazolium chloride; PBST, phosphate-buffered saline and Tween 20; PMSF, phenylmethylsulfonyl fluoride; rDNA, DNA-encoding ribosomal RNA; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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*Correspondence to: James S.C. Gilchrist, Division of Stroke and Vascular Disease, St. Boniface General Hospital Research Centre, Room 4024. 351 Taché Avenue, Winnipeg, Manitoba, R2H 2A6, Canada.

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helix-loop-helix transcription factors, [Onions et al., 1997; Hermann et al., 1998], (b) Ca^{2+}/calmodulin-dependent phosphatases and kinases [Ghosh and Greenberg, 1995], and (c) DREAM, a Ca^{2+}-dependent transcriptional repressor protein [Carrion et al., 1999]. For all of these proteins, Ca^{2+}-dependent control would be expected to occur at the nanomolar-free Ca^{2+} concentrations ($[Ca^{2+}]_F$) reported in resting cells [al Mohanna et al., 1994]. However, nucleoplasmic $[Ca^{2+}]_F$ is independently regulated by perinuclear membrane Ca^{2+} transport mechanisms [Abrenica and Gilchrist, 2000], and very large increases in nucleoplasmic $[Ca^{2+}]_F$ above cytosolic levels have been observed in stimulated cells [Brini et al., 1993; Bkaily et al., 1997]. This appears to be the case in apoptosis [Abrenica and Gilchrist, 2000], where elevation in nucleoplasmic $[Ca^{2+}]_F$ precedes any change in cytosolic $[Ca^{2+}]_F$ [Ruck et al., 2000]. Indeed, the nucleus is distinguished by an apparently large Ca^{2+}-binding capacity as evidenced by total Ca^{2+} being far in excess of that measured free [Chandra et al., 1994]. Thus, it is relevant to ask whether other nuclear Ca^{2+}-binding proteins exist and if they all necessarily bind Ca^{2+} with high affinity.

The first direct characterization of Ca^{2+}-binding proteins in fractionated nuclei revealed several undetermined proteins with relatively high $^{45}Ca^{2+}$-binding affinity and specificity [Schibeci and Martonosi, 1980]. In our earlier studies, we utilized "Stains-All" staining of SDS–PAGE gels to identify potential Ca^{2+}-binding proteins within fractionated nuclei [Gilchrist and Pierce, 1993; Czubryt et al., 1996]. Protein staining by "Stains-All" arises from dye–dye interactions that depend on anionic spacing density of target macromolecules. At elevated densities exhibited by acidic low- and high-affinity Ca^{2+}-binding proteins, a blue spectral J-state is observed [Green and Pastewka, 1974; Campbell et al., 1983]. This useful approach has led to the identity and localization of calnexin to heart and liver nuclear envelopes [Gilchrist and Pierce, 1993; Czubryt et al., 1996]. With these methods, we have also observed a novel blue-staining 110 kDa-protein (p110) within high-salt extracts from rat liver nuclei [Czubryt et al., 1996; Gilchrist et al., 1997]. In the present study, we detail the purification, identification, and Ca^{2+}-binding characteristics of p110, which is immunologically, biochemically, and structurally homologous to nucleolin.

MATERIALS AND METHODS

Protein Purification

Methods for preparation of rat liver nuclei were exactly as we have described [Gilchrist and Pierce, 1993]. To obtain high-salt nuclear protein extracts, highly purified nuclei were resuspended (at 10 mg/ml) in Buffer A containing (in mM) 250 sucrose, 50 Tris-HCl (pH 7.4), 50 KCl, 1 dithiothreitol (DTT), 1 phenyl methyl sulfonyl fluoride (PMSF), 0.01 leupeptin, and then digested (30 min at 4°C) with 250 μg/ml each of DNaseI and RNaseA (Worthington Biochemicals, Freehold, NJ). Two molar NaCl was then added to a final 1.6 M concentration followed by centrifugation (30 min at 4°C and 25,000*g*) to separate nuclear ghosts from high-salt soluble proteins in the supernatant. To separate acidic proteins from histones and globulin-type proteins within high-salt extracts, we then performed ammonium sulfate fractionation. Pelleted acidic proteins obtained from a 40–75% ammonium sulfate cut were resuspended to ~10 mg/ml in and dialyzed against Buffer A containing 5 mM $MgCl_2$ (i.e., Buffer B) for 18 h in SpectraPor (Spectrum Medical Industries, LA) tubing (mol. wt. cut-off 6,000–8,000). Soluble protein was then loaded onto a 5-ml Bio-Rad High-Q Econo-Pac cartridge pre-equilibrated with Buffer C (50 mM Tris-HCl (pH 9.5 at 4°C), 1 mM PMSF, 1 mM DTT, 10 μM leupeptin). Following washing with Buffer C, proteins were eluted at 2 ml/min between 0 and 1 M NaCl in Buffer C. Eluted proteins were resolved by mini-gel SDS–PAGE and fractions exhibiting sensitivity to "Stains-All" (see next section) were pooled, diluted with three volumes of Buffer D (40 mM NaCl, 50 mM Tris-HCl (pH 9.5), 1 mM PMSF, 1 mM DTT, 10 μM leupeptin) and loaded onto a 4-ml heparin-Sepharose column pre-equilibrated with Buffer D. Proteins were eluted with 20 ml of a 40 mM to 1 M NaCl gradient in Buffer D and "Stains-All"-sensitive proteins, were pooled and concentrated to ~10 mg/ml. For comparative Ca^{2+} binding studies, we also purified calsequestrin from rabbit skeletal muscle using essentially identical methods to that described by Slupsky et al. [1987]. Protein was assayed using a modified Lowry procedure [Harrington, 1990].

SDS–PAGE of Proteins

Proteins were separated by denaturing SDS–PAGE in either 1-mm thick 3–13% gradient

gels (Bio-Rad Protean II system) or 10% mini slab gels (Bio-Rad Mini Protean II system) using Laemmli [Laemmli, 1970] buffer systems exactly as we have described [Gilchrist and Pierce, 1993]. Following electrophoresis, gels were stained with the carbocyanine dye, "Stains-All" (3,3′-diethyl-9-methyl-4,5,4′,5′-dibenzothiacarbocyanine), as described [Campbell et al., 1983; Gilchrist and Pierce, 1993], to identify potential nuclear Ca^{2+}-binding proteins revealed by the presence of blue-staining bands. For two-dimensional electrophoresis, isoelectrofocusing (IEF) was performed in 2.5-mm tube gels and a gel solution consisting of 30% (v/v) acrylamide (1.4 ml), 9.5 M urea (7.1 ml), 20% (v/v) CHAPS (1.0 ml), and an ampholyte mix (0.5 ml). The ampholyte mix was a 2:2:1 ratio of the following ampholytes (3–5):(4–6):(3.5–10), respectively. Proteins were prepared at a 1:1 volume by urea denaturation in 9.5 M urea (860 μl), 20% (v/v) CHAPS (40 μl), 2-mercaptoethanol (50 μl), and ampholyte mix (50 μl). Protein IEF was performed overnight at 400 V, with a catholyte of 0.1 M NaOH and anolyte of 0.06% H_2PO_4. Electrophoresis in the second dimension was performed via SDS–PAGE.

$^{45}Ca^{2+}$ and Ruthenium Red Overlays

Nuclear proteins resolved by 3–13% SDS–PAGE gels (see above) were blotted onto nitrocellulose sheets using the Bio-Rad Trans-Blot system and Towbin's buffer [Towbin et al., 1992], as we have described [Gilchrist and Pierce, 1993]. $^{45}Ca^{2+}$ overlays were performed by incubating nitrocellulose blots in 60 mM KCl, 5 mM $MgCl_2$, 10 mM Imidazole (pH 6.8), and 1 μCi/ml $^{45}Ca^{2+}$ at room temperature for 10 min, as described [Maruyama et al., 1984]. Blots were then rinsed twice with distilled de-ionized water and then air-dried for several hours followed by overnight exposure to Kodak X-Omat film. For ruthenium red overlays, blots were washed and then incubated in 60 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.5), and 25 μg/ml ruthenium red as described [Charuk et al., 1990]. Following the appearance of red staining bands, blots were immediately photographed. Control blots were treated identically except for the inclusion of 50 mM Ca^{2+} to competitively inhibit ruthenium red binding.

Partial Amino Acid Sequencing

N-terminal sequencing of heparin-Sepharose purified p110 was performed following excision of intact and 70 kDa peptide fragments from PVDF blots using methods we have previously described [Gilchrist and Pierce, 1993].

Western Blotting

After protein transfer, nitrocellulose membranes were incubated overnight at 4°C in blocking buffer containing 5% (w/v) non-fat dry milk in phosphate-buffered saline and Tween 20 (i.e., PBST). Blots were then incubated in PBST for 2 h with a 1,000-fold dilution of nucleolin-specific R2D2 polyclonal antibody raised against *Xenopus* nucleolin [Heine et al., 1993]. Following brief washing in PBST, blots were then incubated for 1 h with alkaline phosphatase-conjugated anti-rabbit antibody in PBST. Secondary antibody was then decanted and the blot was washed twice in PBST and once in PBS. Conjugated alkaline phosphatase activity was detected with a NBT/BCIP reagent kit supplied by Bio-Rad (Bio-Rad, Hercules, CA) and performed according to the manufacturer's instructions.

Spin Dialysis $^{45}Ca^{2+}$ Binding Studies

$^{45}Ca^{2+}$ binding to purified proteins was performed using displacement equilibrium spin-dialysis as described by Ladant [1995]. Proteins of interest were extensively dialyzed at 4°C in Buffer E containing 100 mM KCl, 10 mM Tris-HCl, 0.2 mM $MgCl_2$ (pH 7.4), and then incubated at a final concentration of 1 mg/ml in Buffer E containing 2 μM $^{45}Ca^{2+}$ (50,000 dpm/nmol). Protein (250 μl) was then placed in a Centricon-10 concentrator and 10 μl was spun through at 1,000*g* for 1 min at 25°C in a Model HN-S clinical centrifuge in order to pre-wet the membrane. The flow-through was returned to the top compartment and the mixture was again triturated. A 5-μl aliquot from this upper compartment was removed and radioactivity determined by liquid scintillation methods. The radioactivity in this aliquot represented the total calcium counts. The $^{45}Ca^{2+}$-containing protein solution was then centrifuged as above, and 5 μl of the flow-through was removed for scintillation returning any excess to the upper compartment. The counts in this aliquot represented unbound $^{45}Ca^{2+}$. This procedure was repeated with consecutive 5-μl additions of unlabeled Ca^{2+} up to a final concentration of between 1 and 3.5 mM. Bound Ca^{2+} was calculated from the relationship where free $Ca^{2+} \div$ total $Ca^{2+} = {}^{45}Ca^{2+}$ counts in the filt-

rate ÷ total $^{45}Ca^{2+}$ counts as described [Ladant, 1995].

RESULTS

The initial indication that rat liver nuclei possessed potential Ca^{2+}-binding proteins was obtained from (a) "Stains-All" staining of SDS–PAGE gels (Fig. 1A) and (b) ruthenium red overlays of electroblotted proteins (Fig. 1B). In SDS–PAGE gels (shown here in greyscale), several distinct red- and blue-staining protein bands were observed in intact nuclei above a darkened background of nucleic acid staining (Fig. 1A; lane 1). The most prominent of these was a blue-staining 110-kDa protein (scrolled arrow) seen to partition into 1.6 M NaCl nuclear extracts (Fig. 1A; lane 2). An additional 95-kDa blue-staining protein, previously identified as calnexin [Gilchrist and Pierce, 1993], and an unidentified 150-kDa red-staining protein, both partitioned into nuclear ghosts (Fig. 1A; solid arrows, lane 3). A similar general pattern of staining was also observed in ruthenium red overlays (Fig. 1B), which provide sensitive detection of proteins known to specifically bind Ca^{2+} [Charuk et al., 1990; Gilchrist and Pierce, 1993]. In Figure 1B, several faintly stained bands were visible in intact nuclei (Fig. 1A; lane 1), but none of these was extracted by endonuclease digestion (Fig. 1A; lane 2). Prominent ruthenium red staining was observed in high-salt extracts at 110 and 40 kDa (Fig. 1A; open arrows, lane 3) and in nuclear ghosts major bands where detected at 150 and 95 kDa (Fig. 1A; solid arrows, lane 4) with several fainter bands also observed.

Because we had identified a 110-kDa protein (hereafter referred to as p110) in high-salt extracts that stained both "Stains-All" and ruthenium red, we conducted $^{45}Ca^{2+}$-overlays of fractionated nuclei to determine its potential as a Ca^{2+}-binding protein. Autoradiographs of $^{45}Ca^{2+}$-overlays (Fig. 2A) showed the presence of a prominent 110-kDa $^{45}Ca^{2+}$-binding protein in high-salt extracts (Fig. 2A; solid arrow, lane 3). Some blot-to-blot variation was observed in the number of several faint $^{45}Ca^{2+}$-binding proteins in high-salt extracts. This appears to arise from the fact that acidic proteins tend to transfer right through nitrocellulose membranes. As seen in lane 1, p110 was faintly detectable in intact nuclei and its extraction was resistant to endonuclease digestion (lane 2). To rule out the possibility that p110 may be derived from ER membranes, we then compared $^{45}Ca^{2+}$-binding profiles of ER and nuclear fractions (Fig. 2B). As shown earlier [Gilchrist and Pierce, 1993], ER membranes (Fig. 2A; lane 1) contain four principal $^{45}Ca^{2+}$-binding proteins (Fig. 2A; open arrows) corresponding to endoplasmin (98 kDa), GRP78 (78 kDa), calreticulin (53 kDa), and protein disulfide isomerase (50 kDa). This contrasts

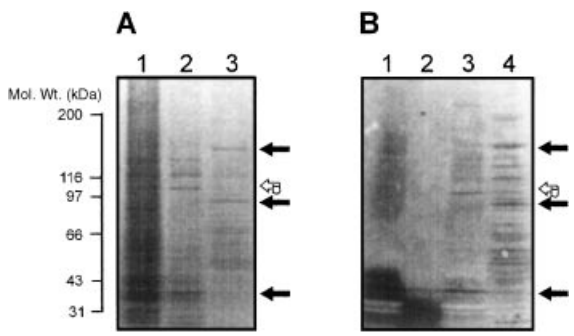

Fig. 1. Nuclear protein staining with ''Stains-All'' and ruthenium red. **A**: ''Stains-All''-stained proteins (50 μg/well) resolved by 3–13% SDS–PAGE in intact nuclei (**lane 1**), 1.6 M NaCl nuclear extracts (**lane 2**), and nuclear ghost proteins (**lane 3**). Molecular weight standards are indicated on the left of the panel. **B**: Ruthenium-red overlay of proteins in intact nuclei (**lane 1**), extracts from endonuclease-digested nuclei (**lane 2**), 1.6 M NaCl nuclear extracts (**lane 3**), and nuclear ghosts (**lane 4**).

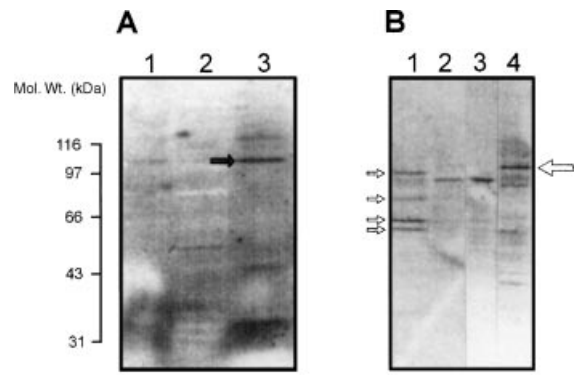

Fig. 2. $^{45}Ca^{2+}$-overlays of nuclear proteins. **A**: Autoradiographs of $^{45}Ca^{2+}$ binding proteins detected by $^{45}Ca^{2+}$-overlays (see the Materials and Methods section) in intact nuclei (**lane 1**), extracts from endonuclease-digested nuclei (**lane 2**), and 1.6 M NaCl nuclear extracts (**lane 3**). **B**: $^{45}Ca^{2+}$-overlay comparing the $^{45}Ca^{2+}$-binding characteristics of ER proteins (**lane 1**), nuclear ghosts from liver (**lane 2**), breast carcinoma cells (**lane 3**), and 1.6 M NaCl nuclear extract proteins (**lane 4**).

with nuclear ghosts where $^{45}Ca^{2+}$ binding was limited to 95-kDa calnexin, as seen in nuclei from liver (Fig. 2A; lane 2) and a breast cancer cell line (Fig. 2A; lane 3). In high-salt extracts (Fig. 2A; lane 4), p110 was the only $^{45}Ca^{2+}$-binding protein (Fig. 2A; open arrow), and it was clearly absent from both ER and nuclear membrane fractions. Thus, p110 is a potential non-membranous nuclear Ca^{2+}-binding protein, which may be novel.

Figure 3 shows the "Stains-All" staining profile of proteins in ammonium sulfate extracts further purified by binding and elution from High-Q columns. In addition to elution of p110 (Fig. 3A; solid arrow), increased purification led to co-elution of a prominent 90-kDa blue-staining band (Fig. 3A; open arrow) along with several faint lower molecular weight bands. The number of bands varied slightly with different preparations, and their presence raised the possibility that they were degradation products of parent p110. That these peptides were undetectable during early salt extractions suggests chromatographic purification removes an important endogenous protease inhibitor. This is supported by the observation that further purification on heparin-Sepharose columns produced a significantly larger spectrum of peptides between 90 and 40 kDa (Fig. 4; open arrows) in addition to p110 (Fig. 4; solid arrow). Staining patterns obtained by all three methods were virtually identical and were unaffected by the presence of protease inhibitors (not shown). This suggests p110 may be autolytically degraded, but peptide fragments may retain Ca^{2+} binding activity.

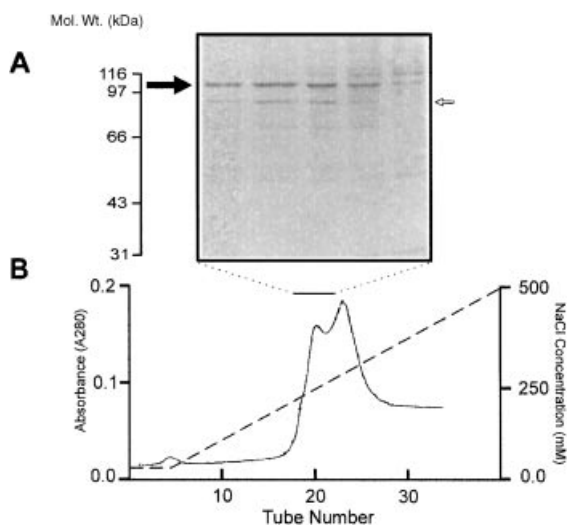

Fig. 3. High Q chromatography of ammonium sulfate nuclear extract proteins. **A**: SDS–PAGE profile of "Stains-All"-sensitive proteins nuclear extract proteins eluted from High Q columns within the elution range indicated by the horizontal bar above Panel B. **B**: Absorbance profile of total eluted protein detected at 280 nm across a 0–500 mM NaCl gradient (dashed line).

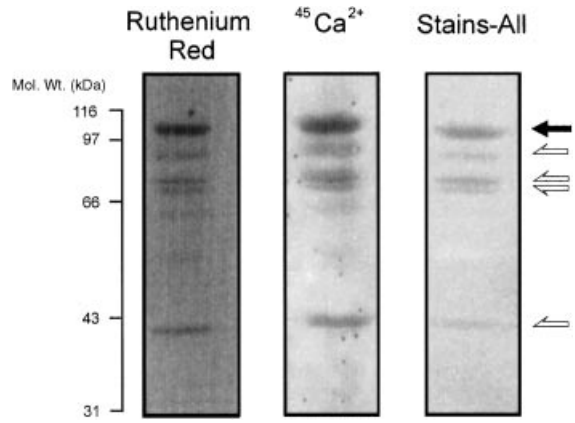

Fig. 4. "Stains-All", ruthenium red, and $^{45}Ca^{2+}$-binding of Heparin-Sepharose purified p110. Purified p110 was resolved on 10% SDS–PAGE gels, and either stained directly with "Stains-All" or electroblotted onto nitrocellulose and overlaid with Ruthenium red and $^{45}Ca^{2+}$ solutions (see the Materials and Methods section). The solid arrow indicates the migration of intact p110. Half arrows indicate apparent p110 autolytic fragments.

In order to determine whether p110 was a true Ca^{2+}-binding protein, we assayed for saturable Ca^{2+} binding via displacement equilibrium spin-dialysis (see the Materials and Methods section). Figure 5 shows p110 Ca^{2+} binding was saturable at 1 mM Ca^{2+} yielding a B_{max} of $\sim$80 nmol Ca^{2+}/mg protein. Ca^{2+} binding to p110 was sigmoidal with half-maximal binding observed at approximately 105 μM Ca^{2+}. These low-affinity Ca^{2+} binding properties are similar to those we reported for calnexin and calreticulin [Gilchrist and Pierce, 1993]. To validate the methods employed, we compared p110 Ca^{2+} binding with the Ca^{2+}-binding properties of purified skeletal muscle calsequestrin, a well-characterized Ca^{2+}-binding protein (see inset, Fig. 5). Here, maximum Ca^{2+}-binding to calsequestrin was approximately 900-nmol Ca^{2+}/mg protein, which is virtually identical to previously reported estimates of 892-nmol Ca^{2+}/mg protein [Charuk et al., 1990]. Thus, these methods appear to reliably reflect Ca^{2+}-binding characteristics of p110. Interestingly, when both proteins were incubated at high Ca^{2+} concentrations, a visible semi-crystalline translucent suspension was formed

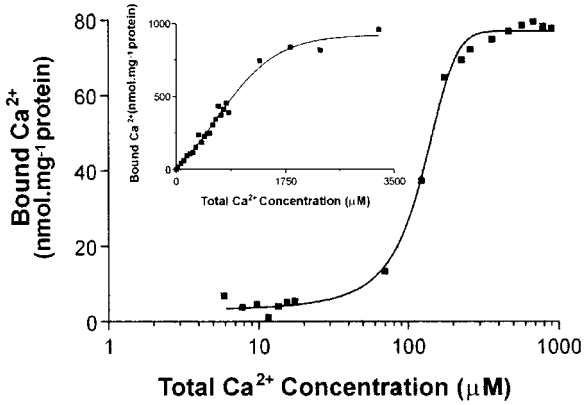

Fig. 5. Saturable $^{45}Ca^{2+}$-binding of heparin-sepharose purified p110. $^{45}Ca^{2+}$ binding to p110 (large panel) is compared to the saturable binding obtained with purified calsequestrin (inset).

(data not shown). For p110, this occurred at approximately 200-μM Ca^{2+}, whereas it occurred around 1 mM Ca^{2+} with calsequestrin. This behavior has been observed earlier with calsequestrin and appears to coincide with major structural changes in the native protein [Williams and Beeler, 1986]. It is possible, therefore, that Ca^{2+} also induces major structural changes in p110.

Although the identity of p110 was initially unknown, we realized its size, acid-base properties, and susceptibility to fragmentation were similar to that reported for nucleolin [Heine et al., 1993]. Subsequent N-terminal sequencing of intact and fragmented protein then suggested p110 was indeed nucleolin (Fig. 6). Peptide A from intact p110 was 90% homologous to the N-terminal sequence of nucleolin. Peptide B, a 70-kDa p110 fragment, was 83%

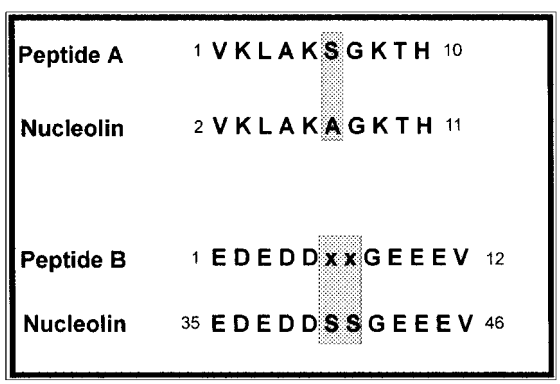

Fig. 6. N-terminal sequencing of p110. Sequence comparison of intact p110 (peptide A) and a 70-kDa peptide (peptide B) with corresponding N-terminal regions of nucleolin. Shaded areas indicated regions of non-homology. Regions marked "x" in peptide B represent unidentified amino acid residues.

homologous to internal nucleolin sequences. The identity of p110 as nucleolin was further corroborated by highly similar staining patterns obtained from both R2D2 immunoblots (see the Materials and Methods section) and "Stains-All" gels (Fig. 7A). As seen in lane 1 (Fig. 7A), R2D2, a highly specific nucleolin antibody [Heine et al., 1993], caused heavy immunostaining staining of intact p110 and faint staining of several peptides between 90 and 50 kDa. Within this range, three corresponding peptide fragments (Fig. 7A; arrowheads) were detected in "Stains-All" gels (Fig. 7A; lane 2). However, lower molecular weight peptide patterns were markedly dissimilar suggesting R2D2 epitopes are different from "Stains-All" binding sites.

To confirm R2D2 immunoreactivity was not due to co-purification of nucleolin with a different 110-kDa blue-staining protein, we performed two-dimensional gel electrophoresis of purified p110 in complementary gels to compare "Stains-All" staining (Fig. 7B) with R2D2 immunostaining (Fig. 7C). Figure 7B shows a single peptide at 110-kDa stained with 'Stains-All" and exhibited a pI of 5.5, similar to that previously reported for nucleolin [Gotzmann et al., 1997]. Subsequent Coomassie Blue staining of this gel showed this peptide to be the only 110-kDa protein (not shown). Figure 7C shows the presence of a single 110-kDa immunostaining peptide with an identical pI of 5.5. Thus, p110 was identical to nucleolin. Note in Figure 7B that several lower molecular weight "Stains-All"-sensitive peptides possessed a lower pI than parent, p110/nucleolin. This general net increase in peptide acidity suggests p110/nucleolin autolysis occurs principally by cleavage of basic residues. In nucleolin, these are clustered at the C-terminal indicating that the acidic N-terminal remains relatively intact. Although not well seen in the photographic image in Figure 7C, immunostaining of the lower molecular weight peptides corresponding to the blue staining bands in Figure 7B could be detected by the naked eye in the original blot.

DISCUSSION

In this study, we identified a 110-kDa Ca^{2+}-binding protein (p110) in 1.6 M NaCl extracts of endonuclease-digested whole rat liver nuclei. Its presence was initially determined from blue

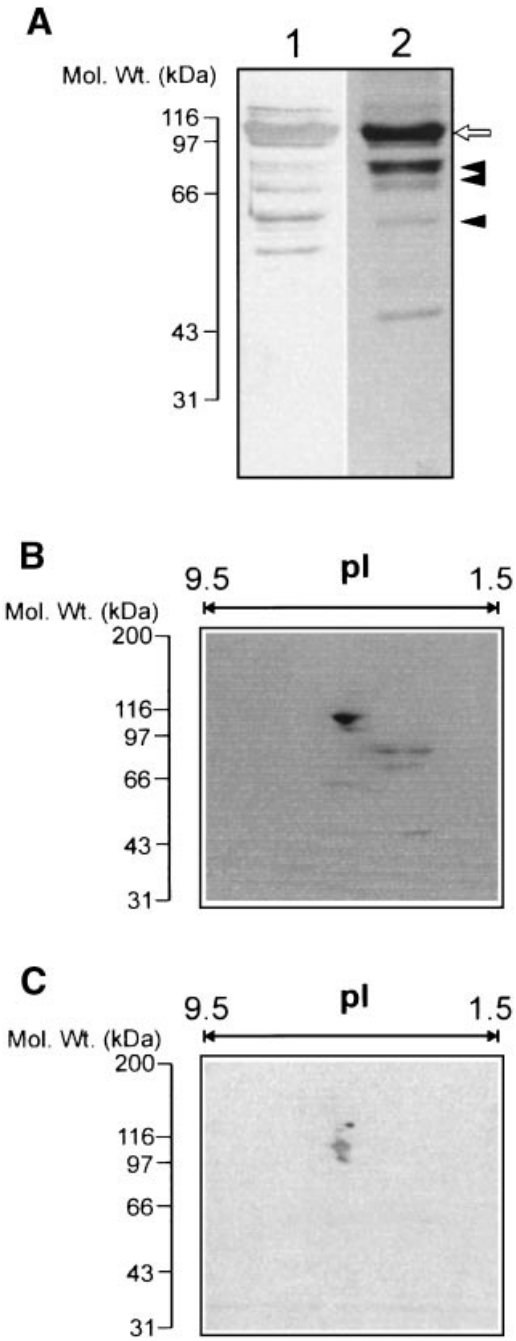

Fig. 7. Comparison of ''Stains-All''- and R2D2 immunostained-stained p110 resolved by one- and two-dimensional electrophoresis. **A**: R2D2 immunostaining (**lane 1**) and ''Stains-All'' staining (**lane 2**) of heparin-Sepharose purified proteins. **Panels B** and **C** represent, respectively, ''Stains-All'' staining and R2D2 immunostaining of purified p110 resolved on two-dimensional IEF gels.

"Stains-All" staining of SDS–PAGE gels and ruthenium red overlays (Fig. 1). As with p110, deep blue staining is shared by several well-characterized Ca^{2+}-binding proteins [Campbell et al., 1983; Gilchrist and Pierce, 1993]. The possibility that p110 might be a Ca^{2+}-binding protein was corroborated with $^{45}Ca^{2+}$ equilibrium binding studies of the purified protein (Fig. 5). Partial N-terminal sequencing of intact and autolytically-fragmented p110 revealed a significant homology (83–90%) to N-terminal sequences found within nucleolin (Fig. 6). This was further corroborated by strong immunoreactivity of highly specific *Xenopus* nucleolin antiserum (R2D2) toward intact and fragmented p110 (Fig. 7). Thus, our study confirms nucleolin is a Ca^{2+}-binding protein and supports a recent observation [Sorokina and Kleinman, 1999] that nucleolin-related protein (NRP), isolated from surface apical membranes of inner medullary collecting duct cells, binds $^{45}Ca^{2+}$ in overlay experiments.

Nucleolin is an intriguing phosphoprotein primarily localized to the dense fibrillar region of nucleoli [Bugler et al., 1982; Heine et al., 1993]. However, it also shuttles between the cytoplasm and nucleus as well as localizing to the plasma membrane [Bouvet et al., 1998]. In nucleoli, it accounts for 5% of the total protein and is involved in transcriptional control of ribosomal DNA (rDNA) expression and ribonucleoprotein assembly [Lapeyre et al., 1987; McGrath et al., 1997; Ginisty et al., 1998]. Nucleolin has a true molecular weight of 78 kDa [Bourbon and Amalric, 1990], and possesses a tripartite domain configuration. Its N-terminal domain ($\sim$300 residues) is enriched in four highly acidic glutamate/aspartate regions flanked by clusters of basic residues [Bugler et al., 1987; Lapeyre et al., 1987]. This region accounts for its relatively low isoelectric pH at 5.5 (Fig. 7) and anomalous mobility (105–110 kDa) in SDS–PAGE gels. The central domain consists of four RNA-binding consensus sequences and mediates observed in vitro interactions with DNA and RNA [Ghisolfi Nieto et al., 1996]. The carboxyl domain is structurally complex due to a predominance of glycine, N^G,N^G-dimethylarginine and phenylalanine residues (GAR domain) and is essential for in vitro nucleic binding and targeting to nucleoli in vivo [Heine et al., 1993].

It is probable the acidic N-terminal domain mediates nucleolin Ca^{2+} binding observed in both overlays and equilibrium binding studies. The N-terminus is argyrophilic [Valdez et al., 1995; Morimoto et al., 2001] and accounts for the high correlation between intense silver-

staining properties of nucleoli and rates of preribosomal RNA biosynthesis [Busch et al., 1979; Lischwe et al., 1979; Ochs et al., 1983; Ochs and Busch, 1984; Busch and Vasko, 1988]. Neither the central domain, with alternating stretches of hydrophilic and hydrophobic residues, nor the highly basic COOH-terminus would be expected to support cation-binding activity. This is supported in our study with retention of prominent "Stains-All", ruthenium red staining and $^{45}Ca^{2+}$ binding by fragmented nucleolin (Fig. 4). All fragments detected by "Stains-All" exhibited markedly lower pI values than parent nucleolin (Fig. 7B). The most acidic of these was an apparent 40 kDa limit peptide (pI = 3.5) suggesting almost complete loss of C-terminal basic residues. Autolysis is an established feature of both native and recombinant nucleolin [Chen et al., 1991; Warrener and Petryshyn, 1991; Fang and Yeh, 1993; Heine et al., 1993], and although exact cleavage sites have not been mapped, our data now provides firm support for an earlier proposal that this occurs primarily in its terminal two-thirds [Fang and Yeh, 1993]. Our observations further suggest Ca^{2+} binding is an intrinsic property of the N-terminal domain, independent of the COOH-terminus, since significant $^{45}Ca^{2+}$ and ruthenium red binding was retained in the 40-kDa peptide fragment (Fig. 4).

Our data indicate that nucleolin is a low-affinity Ca^{2+}-binding protein able to bind 5–6 moles Ca^{2+} per mole of protein (Fig. 5). This resembles the Ca^{2+}-binding properties of many annexins, but differs from those of calsequestrin (Fig. 5; inset), which exhibits almost an order of magnitude higher capacity ($\sim$40 moles Ca^{2+}/mole protein). Thus, it is unlikely that nucleolin functions primarily as a Ca^{2+} storage protein. Deducing Ca^{2+}-dependent functions of nucleolin is difficult because of the broad and highly varied range of activities attributed to it. Nucleolin is an especially versatile protein with a critical role in cellular proliferation and growth [Srivastava and Pollard, 1999]. It possesses both DNA/RNA helicase [Tuteja et al., 1995] and DNA-dependent ATPase activities [Miranda et al., 1995], and binds a wide range of proteins important for rDNA transcription [Tuteja and Tuteja, 1998]. To date, none of these nuclear activities has been shown to be Ca^{2+} dependent. Outside of the nucleus, nucleolin appears to act as a cell surface receptor for viruses [de Verdugo et al., 1995; Qiu and Brown, 1999] and lipoprotein [Semenkovich et al., 1990] with the nucleolin homologue, NRP, able to form cytoskeletal complexes on apical membranes of cultured renal tubular cells [Sorokina and Kleinman, 1999]. In the studies of Semenkovich et al. [1990], ^{125}I-LDL binding to cell surface nucleolin was Ca^{2+}-independent. However, cytoskeletal attachment of NRP was Ca^{2+}-dependent [Sorokina and Kleinman, 1999]. Thus, Ca^{2+} binding may subserve some functions of nucleolin, while others may not require it.

It has been suggested that nucleolin is an "adaptor protein" involved in targeting and anchoring of macromolecules to and within the nucleus, respectively [Srivastava and Pollard, 1999]. Studies suggest the N-terminal domain plays an important role in this by modulating nucleic acid binding as well as influencing protein–protein interactions [Olson et al., 1983; Sapp et al., 1989]. It is conceivable that some of these effects may occur in a cation-sensitive manner. In support of this, 5 mM Mg^{2+} was shown to reduce nucleolin's susceptibility to trypsinolysis through presumed effects upon its quaternary structure to limit accessibility to susceptible lysine residues [Olson et al., 1990]. Our study suggests Ca^{2+} also influences nucleolin ultrastructure through observed formation of translucent semi-crystalline suspensions at approximately 200 μM of total Ca^{2+} (not shown). We observed a similar effect with calsequestrin, and others showed that this coincides with marked conformational transition from an extended rod-like molecule to a compact globular structure [Williams and Beeler, 1986; Slupsky et al., 1987]. It was earlier proposed that the N-terminal acidic domain of nucleolin modulates chromatin decondensation through its interaction with histone H1 in a physical manner similar to that observed with high-mobility-group (HMG) proteins [Erard et al., 1988]. It is possible that Ca^{2+} may modulate nucleolin's influence upon chromatin structure akin to that observed for HMG-1 [Stros et al., 1990, 1994]. Carboxyl terminal sequences of HMG-1, which are structurally homologous to the N-terminal region of nucleolin, promoted DNA looping and compaction at millimolar Ca^{2+} concentrations [Stros et al., 1994].

Future studies are clearly required to elucidate potential Ca^{2+}-dependent functions of nucleolin. Its Ca^{2+} binding characteristics suggests

regulation requires locally high Ca^{2+} concentrations, and under specific conditions these may be present in the nucleus. For example, nucleoplasmic-free Ca^{2+} concentrations ($[Ca^{2+}]_F$) fall between 100 nM and 1 μM [al Mohanna et al., 1994], but total nuclear Ca^{2+} is nearly three orders of magnitude higher [Chandra et al., 1994]. Some locally high $[Ca^{2+}]_F$ may be closely associated with nucleic acids, thus creating a potentially important site of Ca^{2+}-dependent nucleolin activity. Other likely sites may be close to Ca^{2+} pumps and channels on the inner membranes of perinuclear cisterns. It is here, that the majority of nuclear Ca^{2+} is sequestered [Bkaily et al., 1996; Abrenica and Gilchrist, 2000], and significantly, transcriptional activity is high [Duband-Goulet and Courvalin, 2000; Georgatos, 2001]. It may also be possible that Ca^{2+} serves some functions of nucleolin in apoptosis [Pasternack et al., 1991; Brockstedt et al., 1998; Martelli et al., 1999, 2000; Morimoto et al., 2001; Xu et al., 2001]. Its exact role is uncertain, although nucleolin has been suggested to mediate nucleo-cytoplasmic shuttling of Granzyme A during apoptosis [Pasternack et al., 1991]. During apoptosis, whole cell $[Ca^{2+}]_F$ becomes elevated [Nicotera and Orrenius, 1998; Hajnoczky et al., 2000], and the magnitude of increase is particularly large in the nucleus [Abrenica and Gilchrist, 2000]. These conditions may promote nucleolin protein complex formation in the nucleus, in a manner similar to that reported to occur with NRP at surface membranes [Sorokina and Kleinman, 1999].

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