Localization of the Cyclic ADP-ribose-dependent Calcium Signaling Pathway in Hepatocyte Nucleus

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CD38 is a type II transmembrane glycoprotein found on both hematopoietic and non-hematopoietic cells. It is known for its involvement in the metabolism of cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate, two nucleotides with calcium mobilizing activity independent of inositol trisphosphate. It is generally believed that CD38 is an integral protein with ectoenzymatic activities found mainly on the plasma membrane. Here we show that enzymatically active CD38 is present intracellularly on the nuclear envelope of rat hepatocytes. CD38 isolated from rat liver nuclei possessed both ADP-ribose cyclase and NADase activity. Immunofluorescence studies on rat liver cryosections and isolated nuclei localized CD38 to the nuclear envelope of hepatocytes. Subcellular localization via immunoelectron microscopy showed that CD38 is located on the inner nuclear envelope. The isolated nuclei sequestered calcium in an ATP-dependent manner. cADPR elicited a rapid calcium release from the loaded nuclei, which was independent of inositol trisphosphate and was inhibited by 8-amino-cADPR, a specific antagonist of cADPR, and ryanodine. However, nicotinic acid adenine dinucleotide phosphate failed to elicit any calcium release from the isolated calcium stores. The nuclear localization of CD38 shown in this study suggests a novel role of CD38 in intracellular calcium signaling for non-hematopoietic cells.

CD38 is a 42–45-kDa type II transmembrane glycoprotein (1) found in various mammalian tissues and cell types and is capable of converting NAD$^+$ into cyclic ADP-ribose (cADPR)$^1$

(2). This ability is due to the ADP-riboyl cyclase activity found on the extracellular carboxyl domain of the enzyme. The product, cADPR, possesses calcium mobilizing activity independent of inositol 1,4,5-trisphosphate (IP$_3$) (3). Instead, cADPR seems to regulate calcium mobilization through the calcium-induced-calcium release mechanism (4, 5). In addition to cyclizing NAD$^+$ to cADPR, CD38 is also able to use NADP$^+$ as a substrate and catalyze the exchange of its nicotinamide group with nicotinic acid to produce NAADP (5), another potent calcium-mobilizing metabolite.

CD38 is generally believed to be an important surface immunoregulatory molecule; its myriad of possible functions include the induction of B and T cell proliferation (6), regulation of the humoral immune response (7), apoptosis (8), tyrosine phosphorylation of various proteins (9), activation of certain kinases (10), and cytokine release (11). CD38 also displays adhesion properties and might possibly mediate a selectin-type adhesion between different blood populations and human vascular endothelial cells via its putative ligand, CD31 (12). In addition to its immuno-functions, CD38 has been shown to play an important role in insulin secretion via the cADPR-dependent calcium signaling pathway (13). Indeed, results suggest the appearance of autoantibodies to CD38 in patients may contribute to the development of noninsulin-dependent diabetes (14).

The calcium stores mobilized by cADPR and NAADP have been shown to be co-purified with that sensitive to IP$_3$, which is believed to be the endoplasmic reticulum (3, 15, 16). Increasing evidence suggests that the nuclear envelope may also be an important source of Ca$^{2+}$ stores. Thus, calcium transients generated around the nucleus have been shown to be important in various cellular functions including the regulation of cell division (17), gene transcription (18), and nuclear envelope breakdown (19).

It has been shown previously that nuclear envelope isolated from mouse liver cells is responsive to cADPR (20). An important unresolved question is how an ectoenzyme like CD38 with its catalytic site facing the extracellular environment could transport its product, cADPR, into the cells to exert its calcium mobilizing properties. In this study, we show that CD38 is not exclusively an ectoenzyme but, instead, is also an integral protein of the inner nuclear envelope in hepatocytes. We further demonstrate that cADPR but not NAADP is able to release

IP$_3$, inositol trisphosphate; RyR, ryanodine receptor; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; BSA, bovine serum albumin; NAADP, nicotinic acid adenine dinucleotide phosphate; ConA, concanavalin A.
Ca\(^{2+}\) from the nuclear stores independently of IP\(_3\). All the components of the Ca\(^{2+}\)-signaling pathway mediated by cADPR are thus present and operational in the hepatocyte nucleus, suggesting an important functional role in the nuclear environment.

**EXPERIMENTAL PROCEDURES**

**Materials**—Nitrocellulose membrane, alkaline phosphatase-conjugated goat anti-rabbit IgG, 5-bromo-4-chloro-3-indolyl phosphate \(\rho\)-toluidine, \(\rho\)-nitroblue tetrazolium chloride, and hydroxyapatite were obtained from Bio-Rad. Fura-2 and the calcium calibration buffer kit were obtained from Molecular Probes (Eugene, OR). ATP, cADPR, NAD\(^{+}\), NAADP, NGD\(^{1}\), 8-NH\(_2\)-cADPR, copper-imidodiacetic acid-agarose, \(\nu\)-myo-inositol 1,4,5-trisphosphate, and ryanodine were obtained from Sigma. blue Sepharose CL-6B, concanavalin A-Sepharose, and ECL\(^{TM}\) Western blotting detection reagents were obtained from Amersham Pharmacia Biotech. EM-grade goat anti-rabbit immunoglobulin gold conjugate (15 nm) was obtained from British Biocell International (Cardiff, UK). Standard analytical grade laboratory chemicals for the preparation of general reagents were obtained from BDH (Poole, UK), Merck, and Sigma.

**Antibodies**—Production and characterization of the polyclonal antibody against rat CD38 has been previously described (21, 22). Various other antibodies were pre-mixed from the following sources: Anti-human CD38 (AF18), monoclonal anti-Na\(^{+}\)-K\(^{+}\)-ATPase antibody (clone 9A-5), polyclonal (rabbit) anti-calreticulin, and polyclonal (rabbit) anti-GPR78 (BIP) antibody. The monoclonal anti-nucleoporin p62 was obtained from Transduction Laboratories (Lexington, KY). The polyclonal (rabbit) anti-prohibitin was a kind gift from Mark Corl (Neomarkers Inc., Fremont, CA). Affinity-purified goat polyclonal antibody against type 1 (RyR-1), type 2 (RyR-2), and type 3 (RyR-3) ryanodine receptor (RyR-2) (clone 34-C), monoclonal anti-calnexin antibody (clone CO), including monoclonal against type 1 (RyR-1) and type 2 ryanodine receptor (RyR-2) (clone 34-C), monoclonal anti-Na\(^{+}\)-K\(^{+}\)-ATPase antibody (clone 9A-5), polyclonal (rabbit) anti-calreticulin, and polyclonal (rabbit) anti-GPR78 (BIP) antibody. The monoclonal anti-nucleoporin p62 was obtained from Transduction Laboratories (Lexington, KY). The polyclonal (rabbit) anti-prohibitin was a kind gift from Mark Corl (Neomarkers Inc., Fremont, CA).

**Isolation of Nuclear Fraction**—The rat liver nuclear membrane fraction was isolated as described (23–25). Briefly, freshly removed rat liver was homogenized in 6–8 volumes of a medium containing 1.3 m sucrose, 1.0 m MgCl\(_2\), and 10 m potassium phosphate (pH 7.2). After filtration, the homogenate was centrifuged for 15 min at 1000 \(\times\) g. The pellet was suspended in a minimum volume of the homogenization buffer with a final sucrose concentration of 2.2 m and centrifuged at 100,000 \(\times\) g for 1 h. The final nuclear pellet was suspended in a medium containing 0.25 m sucrose, 5.0 m MgCl\(_2\), and 20 m Tris-HCl (pH 7.2) and centrifuged for 1 h at 100,000 \(\times\) g. The resulting pellet was re-suspended in a protein concentration of 10 mg/ml in a medium containing 50 m M Tris-HCl (pH 7.2), 0.3 m sucrose, 150 m M NaCl, 2 m M EGTA, 20% glycerol, 2% Triton X-100, 2 m M phenylmethylsulfonyl fluoride, leupeptin (10 \(\mu\)g/ml), aprotinin (10 \(\mu\)g/ml), and soybean trypsin inhibitor (50 \(\mu\)g/ml). This was allowed to stand for 2 h on ice with gentle stirring and centrifuged for 30 min at 12,000 \(\times\) g. The supernatant containing solubilized nuclear proteins at the final step was then used as the starting material for its further purification.

**Purification of CD38 from the Nuclear Fraction**—The chromatography steps followed the method of Khoo and Chang (21, 22). Briefly, the solubilized nuclear extract was subjected to a series of column chromatography, in the order of blue Sepharose CL-6B, hydroxyapatite, copper-imidodiacetic acid-agarose, and concanavalin (Con) A-Sepharose columns. The extract was applied to a blue Sepharose CL-6B column that has been equilibrated with equilibration buffer (20 m M HEPES (pH 7.2), 0.2 m M NaCl and 0.1% Triton X-100). The column was then washed with 2 volumes of washing buffer (20 m M HEPES (pH 7.2), 0.5 m M NaCl and 0.1% Triton X-100) and eluted with 2 volumes of elution buffer (20 m M HEPES (pH 7.2), 0.5 m M KSCN and 0.1% Triton X-100). The eluate was then subjected to a hydroxyapatite column that has been equilibrated with equilibration buffer (20 m M HEPES (pH 7.2), 0.1 m M NaCl, and 0.1% Triton X-100). The column was then washed with 2 volumes of washing buffer (20 m M HEPES (pH 7.2), 0.1 m M NaCl, and 0.1% Triton X-100) and eluted with 2.5 volumes of elution buffer (0.1 m M sodium phosphate (pH 7.2), 0.1 m M NaCl, and 0.1% Triton X-100). Bound proteins were eluted with 50 m M sodium phosphate buffer (pH 7.2) containing 0.2 m M imidazole, 0.5 m M NaCl, and 0.1% Triton X-100. The eluate was dialyzed overnight in 10 m M Tris-HCl (pH 7.2) containing 0.1% Triton X-100. The protein sample was then loaded onto the ConA-Sepharose column equilibrated with 20 m M HEPES (pH 7.2), 0.2 m M NaCl, and 0.1% Triton X-100. The column was then washed sequentially with washing buffer A (20 m M HEPES (pH 7.2), 0.1 m M NaCl, and 0.1% Triton X-100) and washing buffer B (20 m M HEPES (pH 7.2), 0.1 m M NaCl, 0.1% Triton X-100, and 0.2% sodium deoxycholate (SDS)). The bound proteins were eluted with 20 m M HEPES (pH 7.2), 0.1 m M NaCl, 0.1% Triton X-100, and 0.5 m M \(\alpha\)-methyl-mannoside (Sigma, St. Louis, MO). The eluates were further pooled for 1 h before infiltration with 30% sucrose in PBS overnight at 4 °C. The rat liver tissues were then frozen at −45 °C using isopentane cooled by liquid nitrogen and embedded in Tissue Tek (Miles). Cryostat sections of 5 \(\mu\)m were cut and placed on gelatin-coated slides. An alternative procedure whereby the unfixed freshly removed organs were snap-frozen in liquid nitrogen-cooled isopentane before cryosectioning re-
sulted in similar staining profiles but with a decrease in autofluorescence level, although the degree of morphological preservation was less than that of the fixed tissue. Sections were incubated with PBS buffer containing 10% non-immune goat serum (Sigma) for 1 h at room temperature. The goat serum had previously been decomplemented by heat inactivation at 56 °C for 30 min. The sections were then heated by floating the sections in preheated retrieval medium for 15 min. The sections were then cooled in the retrieval medium for 15 min. The sections were then incubated in 0.01M PBS (pH 7.4) for 1 h and washed in PBS buffer. For the double-labeling experiment, immunostaining was performed with an optimal dilution of both the rabbit polyclonal antibody against rat CD38 and the monoclonal antibody against Na'-K'-ATPase (Affinity Bioreagents Inc.) added concurrently, and the subsequent steps were identical to that of the single-labeling experiment. The immunofluorescence signal was visualized with goat anti-rabbit Alexa 488-conjugated antibody and goat anti-mouse Alexa 546-conjugated antibody. Both Alexa-conjugated dyes were obtained from Molecular Probes.

The optimal incubation time and dilution of antibodies, defined as the highest dilution producing maximal staining and minimal background, were determined for all batches of antibodies and conjugates. All experiments were repeated at least twice, and slides were made in duplicates. Controls with preimmune serum, non-immune serum, and omission of primary antibody were carried out.

Confocal Microscopy—Confocal microscopy was performed using a Carl Zeiss LSM 410 confocal microscope (Carl Zeiss, Germany) equipped with a x40 Fluor objective (NA = 1.3), x63 Plan-Apochromatic objective (NA = 1.4), and a x100 Fluor objective (NA = 1.3), oil immersion. Excitation of the Alexa 488 fluorescent dye was performed at 498 nm, and the emission signal was collected with a LP515-525 emission filter while the Alexa 546 fluorescent dye was excited at 543 nm and the emission signal was collected with an LP590 filter.

Images were stored and processed with Zeiss software. Optical sections collected using the LSCM at 0.5-μm intervals were subsequently processed with a graphic user interface that allowed the display of a nucleus of interest by cursor and adjustment of a three-dimensional box. Other three-dimensional reconstructions were produced using the program Velocity2 by Images3 (Salt Lake City, UT). The gray scale images of the red and green channels were processed first using a smoothing algorithm and were added separately into the program, which combined them and created the three-dimensional surfaces using gray level iso-surfacing and mask definitions. Background thresholding was used during the final reconstruction to enhance the three-dimensional images, which were then tilted by about 30° so that the membrane surfaces could be better appreciated.

Immunoelectron Microscopy—The rats were perfused in 0.5% glutaraldehyde and 4% paraformaldehyde before processing for electron microscopy (EM) as described previously (32). Briefly, post-osmicated samples were dehydrated in an ascending series of ethanol and embedded in araldite. Ultrathin sections were cut and mounted on Formvar-coated copper grids for immunoelectron microscopy, antigen unmasking was done following the procedure of Stirling and Graff (33). Grids for immunolabeling were incubated in large drops of saturated sodium metaperiodate solution for 1 h at room temperature in a humidified chamber followed by washing in distilled water for 45–60 s. The grids were heated by floating the sections in preheated retrieval medium (0.01 M sodium citrate buffer (pH 6)) maintained at 95–100 °C for 10 min. The sections were then cooled in the retrieval medium for 15 min followed by washing in distilled water for 1 min. After antigen unmasking, the grids were first incubated in 0.01 M PBS (pH 7.4) containing 1% immunoglobulin-free BSA for 10 min. Sections were then incubated overnight (18 h) at 4 °C with the CD38 primary antibody at a dilution of 1:50 (optimization was previously done) in PBS containing 1% BSA and 1% Tween 20. Negative controls with both the preimmune sera and omission of the primary antibody were performed concurrently as well. The grids were washed by floating them on drops of PBS (3 changes, 5 min each), followed by 5 min incubation in PBS with 1% BSA. Bound antibodies were visualized by incubating the sections with EM-grade goat anti-rabbit immunoglobulin gold conjugate (15 nm) at 1:75 dilution with 1% BSA and 1% Tween 20 for 1 h at room temperature. The grids were then washed 3 changes, 5 min each. The grids were stained with uranyl acetate and lead citrate before viewing in a Philips BioTwin CM 120 transmission electron microscope (Philips Electron Optics B.V., Eindhoven, Netherlands).

Marker Enzyme Assays—Sucinate dehydrogenase activity was measured according to the method of Green et al. (34). Basically, the spectrophotometric change in the absorbance at 600 nm, which accompanied the enzymatic reduction of 2,6-dichlorophenolindophenol by succinate, was monitored. The molar extinction coefficient was taken to be 16.1 × 103 cm−1 m−1.

NADPH-cytochrome c reductase activity was assayed according to the method of Dignam and Strobel (35). This assay is based on the spectrophotometric measurement of the increase in absorbance at 550 nm due to the reductase-catalyzed production of reduced cytochrome c using an extinction coefficient of 21 × 103 cm−1 m−1. Na'-K'-ATPase activity was assayed by the release of Pi from ATP according to the method of Paul et al. (36). The incubation medium (1 ml final volume) contained 50 mM Tris-HCl (pH 7.4), 5 mM MgCl2, 130 mM NaCl, 20 mM KCl, 1 mM EGTA, 0.1 mM Tris-ATP with or without 1 mM ouabain at 37 °C. Na'-K'-ATPase was defined as the ATPase activity that could be inhibited by ouabain.

Measurement of Ca2+ Release—Ca2+ loading was done by resuspended the purified nuclear fraction in a standard solution containing 125 mM KCl, 2 mM KH2PO4, 50 mM HEPES (pH 7.0), 4 mM MgCl2, 1 mM CaCl2 and supplemented with an ATP-regenerating system, consisting of 2 mM MgATP, 10 mM phosphocreatine, and 20 units of creatine kinase per ml. The mixture was then incubated for 1 h at room temperature and washed with a buffer containing 125 mM KCl, 2 mM KH2PO4, 60 mM HEPES (pH 7.0), 4 mM MgCl2, 2 mM MgATP, 10 mM phosphocreatine, and 20 units/ml creatine kinase. The final nuclear suspension was adjusted to a protein concentration of 10 mg of protein/ml.

The suspension was then added with Fura-2 at a final concentration of 24 μM. The fluorometric measurement of Ca2+ release was done in a thermostatically controlled cell holder, and the nuclear fraction was stirred continuously for the duration of the experiment. Fluorescence was measured at 510 nm and 470 nm. The ratio of the fluorescence (R, 340/380) was calculated using the FeliX software in a RatioMaster fluorospectrophotometer (Photon Technology International, Brunswick, NJ). The maximum, Rmax, and minimum, Rmin, values of the fluorescence ratio were obtained by the addition of 10 μM ionomycin and 4 mM of Ca2+ or 4 mM of EGTA, respectively. Standard Ca2+-EGTA buffers (Molecular Probes) were used for calibration. All experiments were performed at 37 °C. Calibration of the fluorescent signal in terms of calcium concentration was performed as described (37).

RESULTS

Isolation of a Purified Nuclear Fraction—We adopted a well characterized method for purifying the nuclei of rat liver hepatocytes with minimal contamination from other cellular organelles (23–25). The isolation of nuclei based on the application of a high molarity sucrose concentration in the homogenization medium and followed by higher sucrose concentration in a subsequent centrifugation step has been shown to produce a nuclear preparation with minimal microsomal, mitochondrial, and plasma membrane contamination (23) as well as minimal microsomal nuclear association during the nuclear purification steps (24). Table I shows the results of the marker enzyme assays for the liver nuclear fraction compared with that of the liver homogenate. The activity of NADPH cytochrome c reductase, a microsomal marker enzyme, in the isolated nuclei was less than 5% of the activity in the total homogenate (Table I). Mitochondrial marker enzymes were also minimal as succinate dehydrogenase activity was less than 1% of the total homogenate (Table I). We also observed the absence of any detectable Na'-K'-ATPase in the nuclear fraction, indicating that nucleocytoplasmic content contributed negligibly to the Na'-K'-ATPase activity observed (Fig. 1A) and prohibitin (Fig. 1B), a mitochondrial marker. The fraction was, however, enriched with nucleoporin p62 (Fig. 1C), a constitutive nuclear protein, thus attesting to the fact that our nuclear fraction has been enriched for nuclear components.

The nuclear fractions were also probed with antibody against three dominant endoplasmic reticulum markers consisting of GRP78 (BiP), calreticulin, and calnexin (Fig. 1, D–F). Their presence in the nuclear fraction could be seen clearly, but the...
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**TABLE I**

| Marker enzyme activities in the homogenate and the purified nuclear fraction |
|-----------------------------|--------------------------|-----------------------------|
| Fractions                  | NADPH cytochrome c reductase | Succinate dehydrogenase | Na\(^+\)-K\(^-\)-ATPase |
|                            | Total activity\(^a\) | Specific activity\(^b\) | Total activity\(^a\) | Specific activity\(^b\) | Total activity\(^a\) | Specific activity\(^b\) |
| Liver homogenate           | 89.7 (100) | 9.0 | 1794 (100) | 179 | 448 (100) | 112 |
| Isolated nuclei            | 3.9 (4.3)  | 2.3 | 16.7 (0.93) | 9.7 | N/D (0)   | N/D |

\(^a\) Total activity is measured as nmol \(\cdot\) min\(^{-1}\).

\(^b\) Specific activity is measured as nmol \(\cdot\) min\(^{-1}\) \(\cdot\) mg\(^{-1}\) of protein. Limit of detection is 1 nmol \(\cdot\) min\(^{-1}\) \(\cdot\) mg\(^{-1}\).

The presence of CD38 in the hepatocyte nuclei was further demonstrated by immunohistochemical staining. The polyclonal antibody used has been shown to be specific for rat (21, 22) and murine CD38.\(^2\) This was confirmed in controls using CD38\(^{-/-}\) mice (strain C57BL/6), which showed no staining when the anti-CD38 antibody was used under similar conditions (data not shown).

Phase-contrast imaging of the isolated nuclear fraction showed the preparation to be free of whole cells (Fig. 4A). Counterstaining with Hoescht 33342 (Fig. 4B) further showed that the preparation consisted mainly of intact nuclei and has minimal DNA fragments. The nuclei were immunolabeled with the anti-CD38 antibody (Fig. 4C), indicating the presence of CD38 in the nuclei.

The nuclear localization of CD38 was confirmed in both fixed and unfixed cryostat sections of rat liver. Confocal optical sections showed that >90% of the hepatocytes were immunopositive for CD38 (Fig. 5A). In Fig. 5B, the hepatocyte nuclei were counterstained with propidium iodide to show that CD38 was confined mainly to the periphery of the nucleus, indicating its localization on the nuclear envelope or regions of the endoplasmic reticulum adjacent to it. Very low or no immunoreactivity was observed on the plasma membrane. A low level of granular-patterned autofluorescence could be detected in the hepatocytes, which was also observed in unlabeled control slides (data not shown). An individual nucleus in a series of confocal optical sections was graphically selected by a three-dimensional box and digitally analyzed to show that CD38 was primarily located on the nuclear envelope but not in the nucleoplasm (Fig. 5C). A stereo image constructed from the same series of optical sections clearly showed that CD38 was localized exclusively to the nuclear envelope (Fig. 5D).

Electron microscopic examination of the rat hepatocyte showed that the colloidal gold immunoreactivity was localized to the nucleoplasmic side of the inner nuclear envelope (Fig. 6, A and B). Since the primary antibody was raised against a polypeptide of 87 amino acid residues consisting of the carboxyl-terminal region of rat CD38 (21, 22), it is likely that the enzymatic site of CD38 that is located in the carboxyl-terminal region is facing the nucleoplasm whereby it would have access to NAD\(^+\) located therein. No immunoreactivity was observed on the outer nuclear envelope or the endoplasmic reticulum regions adjacent to it. Negative CD38 immunoreactivity was observed with incubation in either the preimmune sera or omission of the primary antibody in the incubation buffer (Fig. 6C) confirmed the specificity of our immunoelectron microscopy results. The results here further corroborate our immunofluorescence data showing that CD38 is localized exclusively to the nuclear envelope.

**Developmental Expression of CD38**—In 2–3-month-old rats, expression of CD38 in the nuclei of the hepatocytes was consistently seen (Fig. 7C). However, in fetuses from the 20th day of pregnancy there was minimal or no expression of CD38 in the hepatocytes (Fig. 7A). In the 1-week-old postnatal rats

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\(^2\) C. F. Chang, unpublished observations.
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Purification of CD38/ADP-ribosyl cyclase from the rat liver nuclear fraction

| Purification step     | Total protein (mg) | Total activity (nmol min$^{-1}$) | Specific activity (nmol min$^{-1}$ mg$^{-1}$) | Fold | Yield (%) |
|----------------------|--------------------|----------------------------------|-----------------------------------------------|------|-----------|
| Solubilized extract  | 120.0              | 45.00                            | 0.37                                          | 1    | 100       |
| Blue Sepharose eluate| 11.10              | 28.80                            | 2.60                                          | 3    | 64        |
| HTP eluate           | 3.00               | 21.60                            | 7.20                                          | 7    | 48        |
| Cu$^{2+}$-IDA eluate | 0.47               | 15.75                            | 33.50                                         | 91   | 35        |
| ConA eluate          | 0.03               | 7.65                             | 255.0                                         | 690  | 17        |

**FIG. 2** Detection of CD38 from nuclear fraction with Western blotting. The presence of a single 42–45-kDa protein band in the nuclear fraction was detected with the anti-CD38 antibody. Eluate from the concanavalin A-Sepharose column was subjected to 10% (w/v) SDS-PAGE under non-reducing (NR) conditions (0.07 M iodoacetamide in the sample buffer) and reducing (R) conditions (0.04 M β-mercaptoethanol in the sample buffer) and followed by blotting onto nitrocellulose membrane, probed with anti-CD38, and visualized by the alkaline phosphatase method. Total protein loaded for each lane was approximately 10 μg.

(Fig. 7B), not all the nuclei were immunopositive for CD38, and the staining intensity was less compared with the adult rats (Fig. 7C). Again, no staining was observed on the plasma membrane or in the nucleoplasm. This can be clearly seen from Fig. 7D whereby the sections were double-labeled with antibodies against CD38 and Na$^{+}$-K$^{+}$-ATPase. Clear demarcation with regard to the spatial distribution of the two enzymes was seen. In comparing Fig. 7, A and C, we could observe a relative increase in the autofluorescence of the liver tissue, which could be due to a developmental increase in levels of autofluorescent substances including that of NADH, flavins, and lipofuscin. In the 1-week-old postnatal rats (Fig. 7B), we could also observe several sinusoid lining cells, in addition to the hepatocytes, showing immunopositivity toward CD38. The immunopositive signal for sinusoid lining cells was also observed in 2–3-month-old adult rats (data not shown) but was not seen in fetal rats. Our result is in agreement with a previous report showing the absence of CD38 expression in human fetal organs such as the kidney, liver, lung, and lymphoid tissue (40). These results indicate that the expression of CD38 in liver is developmentally regulated. It has been suggested that the marked differences of developmentally regulated expression of a number of hepatic protein markers that occur in fetal and adult hepatocytes might be responsible for the immaturity of some liver functions in the neonate (41). A previous study showing the ineffectiveness of cADPR in mobilizing calcium from internal stores in digitonin-permeabilized chick embryo retina cells (42) gives credence to the possibility that CD38 might have a role in the development process.

**Ca$^{2+}$ Release from Nuclear Stores**—Liver nuclei were first loaded with Ca$^{2+}$ as described under “Experimental Procedures,” which was found to be ATP-dependent (20, 43). Consistent with that reported by Gerasimenko et al. (20), the nuclei responded rapidly to cADPR (Fig. 8A). Subsequent addition of IP$_3$, but not ryanodine, elicited a smaller calcium response. Ryanodine added prior to cADPR induced rapid Ca$^{2+}$ release and blocked cADPR from releasing more Ca$^{2+}$ (Fig. 8B). A further addition of IP$_3$ again managed to elicit a smaller calcium response. These results are consistent with cADPR and ryanodine acting on the same target, which is distinct from the target of IP$_3$. This is further demonstrated in Fig. 7C. The primary addition of IP$_3$ elicited a Ca$^{2+}$ response but failed to block a larger Ca$^{2+}$ release activated by subsequent addition of ryanodine. The nuclei became refractory after the addition of ryanodine and failed to respond to cADPR (Fig. 8C). A normal response was seen, however, if cADPR was added before ryanodine, even in the presence of IP$_3$ (data not shown). In all the experiments, the amount of Ca$^{2+}$ released by either 10 μM cADPR or 5 μM ryanodine was consistently measured to be approximately twice that released by 5 μM IP$_3$ (Fig. 8, A–C).

The dose-response experiment of cADPR-dependent Ca$^{2+}$ mobilization showed that the addition of 2 μM cADPR and 10 μM cADPR resulted in a graded increase of Ca$^{2+}$ release from...
DISCUSSION

CD38 is a lymphocyte surface antigen originally defined by monoclonal antibody typing (45). Its expression in lymphocytes is stage-specific and has thus been widely used as a marker for lymphocyte differentiation (46, 47). Subsequent studies (5, 48) demonstrate that it is a novel multifunctional ectoenzyme capable of not only synthesizing cADPR but also hydrolyzing it. The ecto location of CD38, however, raises an unresolved issue of how the product, cADPR, synthesized by the ecto-CD38 can exert its calcium mobilizing activity intracellularly (49).

One model that has been proposed to answer this conundrum involved the internalization of surface CD38, which has been shown to occur upon the addition of various stimuli including that of NAD$^+$ (50) and NADP$^+$ (51). However, this mechanism would be a relatively slow and inefficient method for causing calcium release from intracellular cADPR-sensitive stores. It is possible that internalization represents an alternative mechanism of intracellular signaling unrelated to its enzymatic properties and the Ca$^{2+}$ releasing properties of cADPR. The study by Funaro et al. (52) where they showed that the internalization step is likely to be a negative feedback control mechanism that interrupts signal transduction processes mediated by the surface membrane CD38 gives credence to the above-mentioned possibility.

Recently, it was postulated that transmembrane juxtaposition of two or four CD38 monomers is able to generate a catalytically active channel for selective formation and influx of cADPR to reach cADPR-responsive intracellular calcium stores (53). However, da Silva et al. (54) have shown that there was no direct involvement of ecto-CD38 enzymatic synthesis of cADPR on the regulation of the cADPR-mediated intracellular Ca$^{2+}$ signaling in T-lymphocytes. In addition, the same study has shown that there was no increase of intracellular cADPR when the intact cells were incubated with NAD$^+$. The results described in this study provide a possible resolution to this topological issue. Here we show that CD38 can also be an intracellular enzyme. Its localization in the nucleus of hepatocytes represents the first demonstration of selective association of CD38 with an intracellular organelle. Over 20 years ago, Tamulevicius et al. (55) and Fukushima et al. (56) had independently shown the presence of an unidentified NADase in the nuclear envelope of mouse and rat hepatocytes, respectively. The fact that we identified this NADase in the nuclear envelope as likely to be CD38 suggests that the subcellular distribution of this molecule is more complex than originally thought.

In addition to CD38, we also demonstrated that the isolated liver nuclei also possess the cADPR-sensitive calcium release mechanism. Therefore, all the components of the Ca$^{2+}$-signaling pathway mediated by cADPR are present and functional in the nucleus of the hepatocytes. Our results are in agreement with those reported by Gerasimenko et al. (20) but contrast those by Lilly and Gollan (57), which suggested that the hepatocytes are not responsive to cADPR. The present study and that by Gerasimenko et al. (20), both focused on the hepatocyte nuclei, whereas Lilly and Gollan (57) used mainly liver microsomes. In view of the selective localization of the cADPR system in the nucleus, the negative result observed with the micro-

The nuclear stores (Fig. 8D), 8-NH$_2$-cADPR has been shown to be a potent antagonist of cADPR (44), and Fig. 8E shows that prior addition of 100 $\mu$M 8-NH$_2$-cADPR effectively blocked the subsequent release of calcium by cADPR. Addition of ADP-ribose as a control does not result in Ca$^{2+}$ mobilization from the nuclear stores (data not shown). However, unlike cADPR, NAADP has no effect on Ca$^{2+}$ mobilization from the nuclear envelope stores (Fig. 8, F and G). In Fig. 8F, the addition of cADPR caused a Ca$^{2+}$ response, but further addition of NAADP (100 nM to 100 $\mu$M) in the final concentration failed to elicit any further response. Conversely, the primary addition of NAADP failed to elicit any Ca$^{2+}$ mobilization from the nuclear Ca$^{2+}$ stores, but the subsequent addition of cADPR again caused the Ca$^{2+}$ mobilization response as observed previously (Fig. 8G).

Addition of 1 mM NAD$^+$ resulted in a slow and steady release of Ca$^{2+}$ from the nuclear stores (Fig. 8H). This result suggests that cADPR could be produced from NAD$^+$ in the nucleus, and the cADPR, in turn, was able to act on the cADPR-sensitive Ca$^{2+}$ stores. Taken as a whole, our results indicate the existence of a cADPR-sensitive Ca$^{2+}$ release mechanism in the nuclear envelope of rat hepatocytes. The sensitivity of the mechanism to ryanodine suggests the existence of ryanodine-sensitive Ca$^{2+}$ release channels, which could be regulated by cADPR in the nuclear envelope of rat hepatocytes.

**Fig. 4. Localization of CD38 to isolated liver nuclei.** A, phase contrast microscopy. B, nuclei stained with Hoechst. C, the same slide as in B was immunostained with anti-CD38. Scale bar, 10 $\mu$m.
somes indicates the possibility of a cADPR-sensitive calcium pool located exclusively in the nuclei.

Another important aspect of nuclear Ca\(^{2+}\) signaling that might be regulated by CD38 is the fact that, apart from cADPR, it also produces NAADP that has previously been shown to possess Ca\(^{2+}\)mobilizing properties as well (5). Recently, it was shown that NAADP is able to mobilize calcium from pancreatic acinar cells and rat brain microsomes in an IP\(_3\)- and cADPR-independent manner (16, 58). However, our results show that NAADP is not able to mobilize Ca\(^{2+}\) from rat hepatocyte nuclear calcium stores. This observation suggests the possibility that both the nuclear and endoplasmic reticulum calcium stores are physiologically regulated in a different manner.

The nuclear CD38 in hepatocytes can cyclize NGD\(^{+}\) to cG-DPR, a fluorescent analog of cADPR (Fig. 3B). This characteristic clearly distinguishes it from regular NADases, which hydrolyze NGD\(^{+}\) to a nonfluorescent product, GDP-ribose (59). However, the hepatocyte CD38, similar to that found in other systems (2, 60–62), also possesses NADase activity (Fig. 3B).

CD38 has been shown to be present on the surface of many cell types (5, 48). Indeed, we have found that the plasma membrane fraction isolated from hepatocytes contained abundant GDP-ribosyl cyclase activity (63). Upon using a different polyclonal antibody against CD38, we observed high immunoreactivity on the sinusoidal domain of rat hepatocytes but, surprisingly, the same antibody did not cross-react with the nuclear CD38 (63). It is possible that a different isoform or post-translationally modified form of CD38 is present on the nuclear envelope of the rat hepatocyte as compared with the plasma membrane CD38. This is not without precedence. For example, multiple IP\(_3\) receptor isoforms have been shown to be present both on the plasma membrane and internal membranes (64, 65).

It is also interesting to note that from the data of our purification steps (Table II) and the size of the protein calculated from the result of the immunoblot (Fig. 2), the nuclear CD38 appears to be glycosylated as it apparently binds to ConA-Sepharose, whereas its molecular weight is similar to that of glycosylated surface membrane CD38. ConA is a lectin, which binds reversibly to molecules with α-ᴅ-mannopyranosyl, α-ᴅ-
CD38 was localized to the inner membrane (indicated by arrows) of the nuclear envelope. Ultrathin sections after antigen unmasking were immunostained with anti-CD38 primary antibody and secondary antibody conjugated with 15-nm gold particles. A and B, CD38 was localized to the inner membrane (indicated by arrows) of the nuclear envelope. C, control section with no gold particles observed. C and N denote the cytoplasm and nucleus, respectively. Scale bar, 100 nm.

Fig. 6. Ultrastructural localization of CD38 to the nuclear envelope in rat hepatocyte nucleus. Ultrathin sections after antigen unmasking were immunostained with anti-CD38 primary antibody and secondary antibody conjugated with 15-nm gold particles. A and B, CD38 was localized to the inner membrane (indicated by arrows) of the nuclear envelope. C, control section with no gold particles observed. C and N denote the cytoplasm and nucleus, respectively. Scale bar, 100 nm.

Fig. 7. CD38 in the rat liver at different stages of development. Confocal images of fixed liver sections were obtained by exciting at 488 nm and collecting from two channels, with the green filter (BP515–525) to obtain the Alexa 488 signal (anti-CD38) and the red filter (LP590) to obtain the autofluorescence in order to highlight the liver morphology. Liver section of fetal rat at the 20th day of pregnancy shows that the hepatocytes did not express CD38 (A), whereas the liver section of 1-week postnatal rat shows that CD38 was expressed in the nuclear region of the hepatocytes (B). In addition, several sinusoidal lining cells (white arrows) were also immunopositive for CD38. C, liver section of a 3-month-old adult rat shows an increase in staining intensity for CD38 in the nuclear region. An increase in the autofluorescence of the hepatocytes is also seen. D, liver sections were doubly labeled with anti-CD38 (green) and anti-Na+-K+-ATPase (red). The figure on the left shows a single optical section. The figure on the right is a representation of a three-dimensional reconstruction based on a series of optical sections. The level of autofluorescence was minimized by lowering the laser intensity and using unfixed snap-frozen liver. A–C, scale bar, 50 μm. D, scale bar, 10 μm.

glucopyranosyl, and sterically related residues. Our results indicate that nuclear CD38 contains similar glycoconjugates that are able to bind ConA. The presence of glycoproteins localized to the nuclear envelope is not surprising as Willem et al. (66) have previously shown that the nuclear envelope of rat acinar cells was selectively labeled for ConA, thereby indicating the presence of specific glycoproteins with an affinity for ConA in the nuclear envelope.

The fact that CD38 is transported to the surface membrane with its carboxyl-terminal domain containing the enzymatic site facing the extracellular region suggests that the transport to the nuclear envelope would result in the luminal localization of the CD38 enzymatic site. However, our immunoelectron microscopy studies allowed us to identify the localization of CD38 to the inner nuclear envelope with its catalytic site facing the nucleoplasm. Currently, the precise mechanism of protein transport to the inner nuclear envelope is not known. Complemented with the fact that the outer membrane of the nuclear envelope has different characteristics including different protein composition from the inner nuclear membrane, it is possible that the pathway for the distribution of CD38 to the nucleus is distinct from that of the pathway to the surface membrane.

Our results indicate that the Ca2+ release mechanism is probably located on the inner nuclear membrane. This would allow the release of calcium into the nucleus where it can act directly to activate Ca2+-sensitive nuclear events. In fact, this appears to be the case in starfish oocyte where it was shown that the introduction of cADPR directly into the nucleus is able to activate calcium changes in the nucleoplasm (67). Furthermore, Gerasimenko et al. (20) have shown that both IP3 and cADPR can cause the release of calcium from the nuclear envelope into the nucleoplasm where it can also diffuse out through the nuclear pores.

NAD-pyrophosphorylase, an enzyme that catalyzes NAD+ formation from nicotinamide mononucleotide and ATP, is found predominantly with an intranuclear localization (68). Thus, NAD+ produced locally could be a source of substrate for the ADP-ribozyme cyclase activity of CD38. In any case, there should be a mechanism to regulate the cADPR production. It is likely that there is a mechanism whereby CD38 is inactive until stimuli trigger it to an active state, and cADPR is produced. This is supported by the fact that a previous study has shown that the NADase in the nuclear envelope is present in a latent form (55). In addition, Sato et al. (69) have recently discovered a novel peptide inhibitor to human BST-1/CD157. This cADPR-synthesizing enzyme and CD38 are believed to have evolved from a common ancestor by gene duplication (70). It is not inconceivable that there exists similar inhibitor peptides or proteins to regulate the intracellular activity of CD38 as well.

It is generally believed that small molecules such as cADPR or its precursor, NAD+, can freely diffuse through the nuclear pore complex due to the fact that the nuclear pore complex supposedly allows the permeability of the nuclear envelope to all molecules with masses as large as 30–60 kDa (71). In addition, Oishi and Yamaguchi (72) have shown that liver cytosolic NAD+ is a factor in the regulation of the nuclear Ca2+ concentration. Thus, the NAD+ present either in the cytosol or in the nucleus should be accessible to the CD38-mediated Ca2+ release mechanism in the inner nuclear membrane.

Another point of interest is the localization of the calcium channels regulated by cADPR. Although cADPR has been shown to be an activator of ryanodine receptors, curiously until now, there have been conflicting reports regarding the nature of ryanodine receptors in the liver. The presence of ryanodine-sensitive Ca2+-induced Ca2+ release pool in the hepatocyte cell has been shown in the study by Osada et al. (73), and another study by Martinez-Merlos et al. (74) has shown via a [3H]ryanodine binding assay that the rat liver contained high levels of ryanodine-binding sites when compared with the liver of five other rodent species. The physiological importance of ryanodine-sensitive Ca2+-induced Ca2+ release pool in the hepatocyte cell...
FIG. 8. Ca\(^{2+}\) release from the nuclear fractions. cADPR, ryanodine, IP\(_3\), 8-NH\(_2\)-cADPR, NAADP, and NAD\(^+\) were added at the times and to the final concentrations as indicated (A–H). D, the dose-response experiment was performed with the addition of 2 and 10 \(\mu\)M cADPR. Representative traces of basal Ca\(^{2+}\) levels and Ca\(^{2+}\) release after addition of the respective concentrations of cADPR were superimposed on a single graph to highlight the graded response of the cADPR-dependent Ca\(^{2+}\) mobilization from nuclear stores. Results shown are representative of five experiments.
dine receptors in the liver is clearly shown in the work of Komazaki et al. (75) whereby they observed that mutant mice lacking both RyR1 and RyR3 showed hypertrophy of the liver and an excessive accumulation of glycogen granules in the hepatic cells.

In contrast, Giannini et al. (76) have reported the absence of messenger RNAs encoding the three known RyR isoforms in liver extracts, and Guirhard et al. (77) reported that the liver nuclei do not possess ryanodine receptors. In our study, we also failed to detect the presence of ryanodine receptors in the rat hepatocyte using two different antibodies against all the known isoforms of ryanodine receptors (data not shown). Immunostaining and Western blot with the anti-ryanodine receptor antibodies failed to yield any positive result while immunoreactivity was observed in the positive control tissues including the heart and skeletal muscle (data not shown). Our observation raises three possible corollaries. One possibility is that there exists a novel isoform of RyR in liver cells, which has yet to be characterized. On the other hand, it is possible that ryanodine and, in turn, cADPR might be mobilizing Ca\(^{2+}\) release from the nuclear stores in a novel manner other than is adjacent to the inner nuclear membrane. Indeed, as a precedent, the presence of functional CD38 localized to the inner nuclear envelope as indicated by past studies (20, 24) as well as by the present study that IP\(^3\) receptors at low densities, which are below the limit of our detection system.

Based on our results, we proposed a model whereby the presence of functional CD38 localized to the inner nuclear envelope is playing a central role in regulating intracellular calcium signaling via its ability to form the potent Ca\(^{2+}\)-mobilizing nucleotide, cADPR (Fig. 9). The diagram illustrates the presence of the two known Ca\(^{2+}\)-signaling pathways on the inner nuclear envelope as indicated by past studies (20, 24) as well as by the present study that IP\(^3\) and cADPR can both cause the transient rise of calcium concentration in the nucleus.

Our study did not allow us to reach a definite conclusion with regard to the precise localization of the cADPR-sensitive Ca\(^{2+}\)-signaling pathway present in the nucleus according to the results presented in this paper. The IP\(^3\)-dependent Ca\(^{2+}\)-signaling pathway known to be present in the nucleus (23) is also illustrated to highlight the fact that multiple Ca\(^{2+}\)-signaling pathways can be found in the nuclear region. The possibility that cADPR might be diffusing through the nuclear pores to act on distant target sites is also shown in the diagram.

![Schematic diagram of the cADPR-dependent Ca\(^{2+}\)-signaling pathway present in the nucleus according to the results presented in this paper.](image)

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