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Insights Into Nuclear Localization and Dynamic Association of CD38 in Raji and K562 Cells

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Abstract CD38 is a type II transmembrane glycoprotein found mainly on the plasma membrane involved in the metabolism of cADPR and NAADP, two nucleotides with calcium mobilizing activity independent of inositol trisphosphate. Recent data report the presence of CD38 in different cellular compartments raising new questions about its effective role in cellular metabolism. In rat hepatocyte nuclei, CD38 has been proposed as a responsive to cADPR integral inner membrane protein suggesting that the nuclear envelope may also be an important source of Ca^{2+} stores. Further reports indicating that CD38 is localized in nuclear compartments in a variety of cell types and tissues including brain, liver, eye, spleen, and bone raise the condition of resolving the question concerning the effective presence of CD38 within the nucleus. Here we report data supporting the presence of CD38 at nuclear level independently of expression of surface CD38. We utilized two different human leukemia cell lines expressing or not expressing CD38 molecule on their cell surface. The morphological and biochemical results including enzymatic activity and proteomic determinations explain the effective nuclear localization of CD38 in human Raji and K562 cells. Since cell nucleus is a complex and highly dynamic environment with many functionally specialized regions, the nuclear localization of specific proteins represents an important mechanism in signal transduction. The presence of CD38 at the interchromatin region whether linked to nuclear scaffold or stored in nuclear structures as micronuclei and Cajal bodies co-localizing with coilin, suggests its involvement in nuclear processes including transcription, replication, repairing and splicing. J. Cell. Biochem. 103: 1294–1308, 2008.

Key words: CD38; nucleus; nuclear bodies; human cell line

Human CD38 antigen, a 42- to 45-kDa type II transmembrane glycoprotein, is structurally composed of a short N-terminal cytoplasmic domain, a single membrane-spanning region and a long C-terminal extracellular catalytic domain [Deaglio et al., 2001a]. Apart from its ability to function as a transmembrane receptor [Ferrero et al., 2000], CD38 is also known to be a bifunctional ectoenzyme which is able to catalyze the production of cyclic adenosine diphosphate ribose (cADPR) and adenosine diphosphate ribose (ADPR) from nicotinamide adenine dinucleotide (NAD^+), and nicotinic acid adenine dinucleotide phosphate (NAADP) using $NADP^+$ as a substrate and catalyzing the exchange of its nicotinamide group with nicotinic acid [Moreschi et al., 2006]. These three products are involved in calcium mobilization by distinct mechanisms: ADP-ribose exploits its activity by binding the plasma membrane TRPM2 cation channel, NAADP induces calcium release from alternate ER

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stores and from RyR in T-lymphocytes [Dammermann and Guse, 2005] and in skeletal muscle [Hohenegger et al., 2002], thus suggesting that several receptors can be targeted by $NAADP^+$, while cADPR acts on Ca^{2+} release through the ryanodine receptor channel (RyRs) present on the endoplasmic reticulum. This channel is activated by Ca^{2+} itself [Lee, 1997], and is known as the Ca^{2+}-induced Ca^{2+} release (CICR) pathway. cADPR increases sensitivity of RyR to Ca^{2+} and thereby regulates Ca^{2+} release through RyRs via the CICR. This mechanism is independent from that of inositol 1, 4, 5-trisphosphate (IP_3).

CD38 is ubiquitously distributed in mammalian tissues. It has been reported to be expressed both on the plasma membrane as well as in intracellular membranes in hematopoietic and non-hematopoietic cells [Deaglio et al., 2001a]. CD38 present on the surface of many cell types acts as a transmenbrane receptor involved in cell-signaling molecule and in different functions, including T proliferation, inhibition of B cells lymphopoiesis, apoptosis, cytokine release and tyrosine phosphorylation of proteins [Kirkham et al., 1994]. 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 [Deaglio et al., 2001b].

Recent reports indicate that CD38 can be expressed in the membranes of subcellular organelles and may gain access to the substrate within these organelles and produces metabolites that regulate calcium homeostasis directly within the organelles [Adebanjo et al., 1999]. The first reports demonstrating that CD38 could be localized within intracellular membranes were performed using cells isolated from brain and heart tissues [Khoo and Chang, 1999]. Further reports indicated that CD38 is localized in intracellular compartments in a variety of cell types and tissues including lung, liver, eye, spleen, osteoclasts and fibroblasts [Khoo and Chang, 1998, 1999, 2002; Ceni et al., 2002]. Although in these studies CD38 has been detected at low levels in organelles like the mitochondria, Golgi and ER, CD38 has been reported to be expressed at the highest levels in nuclear membranes.

Interestingly, in the CNS regions, the nuclear envelope showed constant labeling of CD38 associated to the outer membrane [Yamada et al., 1997]. On the other hand, several studies have reported that CD38 is localized in the inner nuclear membrane [Gerasimenko et al., 1995; Humbert et al., 1996]. At this level, CD38 has been shown to gate Ca^{2+} influx from the nuclear envelope into the nucleoplasm through RyRs, probably to regulate gene expression during bone formation. In contrast, other studies prove that in B murine lymphocytes employing a protocol of mild membrane solubility for the separation of plasma membranes from other intracellular membranes, after immunoprecipitation, CD38 was exclusively detected in the plasma membrane protein containing soluble fraction and not in the insoluble fraction which was highly enriched by nuclear, endoplasmic reticulum and mitochondrial proteins [Moreno-Garcia et al., 2005]. All the previous studies regarding nuclear localization of CD38 have been conducted on animal models, such as rats and mice, but its precise function is still unknown. Moreover the human model remains mainly for clarification.

The aim of the present study is to confirm and detail the presence of this peculiar ectoenzyme at nuclear level in human cells whether expressing or not expressing the CD38 molecule on their cell surface.

MATERIALS AND METHODS

Cell Lines

The human Raji (Burkitt lymphoma, ATCC N.CCL-86) [Pulvertaft, 1964] and K562 (chronic myelogenous leukemia, ATCC N: CCL-243) [Lozzio and Lozzio, 1975] cell lines were maintained in continuous culture in RPMI-1640 medium supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 100 mM Na-pyruvate, 25 mM Hepes and antibiotics. Cells were grown with more than 98% viability as determined by trypan blue exclusion test. At different times of cultures the cells were processed for morphological and biochemical analyses.

Nuclear Preparation

Purified nuclei were obtained by a hypotonic shock combined with non-ionic detergent (10 mM Tris-Cl, pH 7.8, 0.3% Triton X-100), essentially as described by Trubiani et al. [2000]. 0.5 mM phenylmethylsulfonyl fluoride, 10 mM β-mercaptoethanol, 1 mM EGTA, 10 μg/ml leupeptin, 0.3 μM aprotinin, 15 μg/ml calpain I inhibitor, and 7.5 μg/ml calpain II inhibitor

were also added to the buffer (lysis buffer). For each experiment, 10×10^6 cells were suspended in 1 ml of lysis buffer and incubated for 5 min at 0°C. Nuclear supernatant fraction (crude cytoplasm) was precipitated by trichloroacetic acid and solubilised in 200 μl of electrophoresis sample buffer. Nuclear purity was assessed by detection of β-tubulin and only nuclear preparations showing a complete absence of this molecule in Western blot were used.

Immunoprecipitation

This was accomplished as reported elsewhere [Tabellini et al., 2003]. Briefly, protein G-agarose (20 μl) was incubated with goat anti-mouse IgG as bridging specific antibody for 2 h at 4°C. Subsequently the samples were gently shaken-incubated with 5 μg of anti-CD38 antibody (AT1 or IB4) for 1 h at 4°C. Nuclear extracts were pre-cleared by incubation with 20 μl protein G-agarose alone for 1 h at 4°C, prior to the addition of 20 μl anti-CD38-conjugated protein G-agarose antibody complex. The pre-clearing step is usually done for immunoprecipitation because it lowers the amount of non-specific proteins in the lysate and removes proteins that may have a possible non-specific interaction and a high affinity for Protein G. Cleared samples were incubated for 6 h at 4°C, and then centrifuged for 5 min at 3,000*g* at 4°C. Immunoprecipitates were washed four times in 20 mM Hepes–NaOH, pH 7.9, 100 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 0.25% NP-40 and then prepared for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Protein was quantitatively recovered from supernatants of immunoprecipitates by precipitation with 25% trichloroacetic acid. The level of recovered protein was measured according to the instruction of the manufacturer using the Bio-Rad (Hercules, CA) Protein Assay (detergent compatible).

Western Blot Analysis of Immunoprecipitates

Protein separated on SDS–PAGE was transferred to nitrocellulose sheets using a semidry blotting apparatus. Sheets were saturated for 60 min at 37°C in blocking buffer, then incubated overnight at 4°C in blocking buffer (PBS supplemented with 5% NGS and 4% BSA) containing primary antibody (IB4 or AT1). After four washes in PBS containing 0.1% Tween-20, they were incubated for 30 min at room temperature with peroxidase-conjugated secondary antibody diluted 1:5,000 in PBS–Tween-20, and washed as above. Bands were visualized by the ECL method.

Electron Microscopy Analysis

The morphology of the intact cells and nuclear preparations was analyzed after fixing with 1.25% glutaraldehyde in a cacodylate buffer for 30 min and post-fixed with 1% OsO_4 in the same buffer. Samples were stained overnight with saturated aqueous uranyl acetate solution, dehydrated and embedded in Spurr medium. Semithin sections were stained with toluidine blue solution and observed at light microscope level, while thin sections were counterstained with lead citrate. Ultrastructural observations were carried out using a Philips CM10 electron microscope at 60 kV.

Immunoelectron Microscopy

Intact cells were fixed with 4% paraformaldehyde and 0.1 M cacodylate buffer (pH 7.4) for 30 min at 4°C. Samples were dehydrated and embedded in Unicryl at −20°C. Thin sections, mounted on nickel grids, were incubated overnight with the monoclonal anti-CD38 antibody (AT1) at 4°C. After washing with PBS, the sections were incubated for 1 h with rabbit anti-mouse IgG labeled with 20 nm diameter colloidal gold, stained with uranyl acetate, and examined by electron microscopy.

Immunofluorescence Staining and Confocal Laser Scanning Microscope (CLSM) Analysis

We set out a series of experiments aimed at clarifying the subnuclear localization of CD38 in Raji and K562 whole cells and in isolated nuclei. Fixation, permeabilization, and immunostaining were performed at 4°C. Briefly, cells were saturated in PBS containing saponine (0.001%, v/v) for 15 min and washed in PBS. The permeabilized cells and nuclear preparations, obtained as described, were washed in Cacodylate buffer 0.5 M pH 7.4, fixed for 1 h with 2% paraformaldehyde in the same buffer and, then incubated with normal goat serum (in 10 mM PBS, 1:10, pH 7.4, for 15 min) and treated either without antibody, with non-immune mouse IgG, or with monoclonal anti-CD38 antibodies (AT1, IB4) at the concentration of 2 μg/ml. As negative control the samples were also incubated with HLA class I primary antibody instead of anti-CD38. After 2 h of incubation the samples were rinsed with PBS and incubated for 60 min with

goat FITC-conjugated anti-mouse IgG secondary antibody. Subsequently PBS washed samples were stained with a 0.1% PI solution for 10 min. Cells were again washed in PBS mounted with 0.5% *p*-phenylenediamine in 20 mM Tris, pH 8.8, 90% glycerol, sealed, and left to dry before examination. Images were collected through a ZEISS LSM510 META (Jena, Germany), confocal system, equipped with an argon laser and helium-neon source. Optical sections were obtained with a 0.2 μm increment on the z-axis. The green and red digital images, respectively, were merged to detect any overlapping distribution of the two proteins (yellow).

Antibodies, Fixation, and CLSM Analysis of CD38 and Colin Co-Localization

To establish the localization of CD38 in Cajal bodies we performed a double immunostaining of Raji and K562 nuclei using antibodies recognizing CD38 and p80-coilin. Purified nuclei were washed in PBS and fixed for 2 h with 2% paraformaldehyde in Cacodylate buffer 0.5 M pH 7.4. Nuclei were subsequently washed in PBS, incubated with 10% goat serum in PBS for 20 min, followed by incubation with primary antibodies for 4 h. The following antibodies were used: rabbit anti-p80 coilin polyclonal serum (concentration 1 μg/ml); mAb (IB4 or AT1) anti-CD38 (concentration 2 μg/ml). Three washes with PBS were carried out before incubation with secondary antibody affinity-purified Texas red-conjugated goat anti-rabbit and FITC-conjugated goat anti-mouse for 45 min. Samples were washed in PBS mounted with 0.5% *p*-phenylenediamine in 20 mM Tris, pH 8.8, 90% glycerol, sealed, and left to dry before examination. Images were collected through a ZEISS LSM510 META, confocal system, equipped with an argon laser and helium-neon source. Optical sections were obtained with a 0.2 μm increment on the z-axis. To separate emissions of the two fluorochromes HTF 488/543 and NTF 545 primary and secondary dichroic mirrors respectively were used. Detector bandpass filters were set over 505–530 and 565–615 ranges for the green (FITC) and red (Texas red) emissions and simultaneously recorded. In the CD38/p80-coilin co-localization experiments, to prevent any overlapping between the two emissions, acquisition was performed setting the instrument in multi-track mode. Cells had a mean thickness of 2 μm and the image series obtained from the Texas red and FITC signals were electronically merged and pseudo stained. The green and red digital images, respectively, were merged to detect any overlapping distribution of the two proteins (yellow).

Bidimensional Electrophoresis

Total cellular extract and nuclear fractions of Raji and K562 cells were washed with PBS and immediately re-suspended in 0.5 ml lysis buffer (8 M urea, 4% CHAPS, 40 mM Tris base, 2 mM TPB) supplemented with proteinase inhibitors. The samples obtained were sonicated 40 sec twice and incubated with 150 U of endonuclease for 15 min at room temperature. After centrifugation at 12,000*g* the supernatant was assayed for protein content (BCA assay) and stored at −80°C.

A two dimensional gel electrophoresis of each sample was performed using the Immobiline/polyacrylamide system. A quantity of the solubilised protein corresponding to 50 μg (analytical runs) of total protein was used for each electrophoretic run. IEF was carried out on a commercial IPG strip (Amersham) providing a non linear pH 3–10 gradient. The second dimension was carried out in a Laemli system on 10% homogeneous polyacrlamide gel (9.6 × 10.5 cm) at 25 mA/gel. Analytical gel was stained with ammoniacal silver nitrate. Gel images were digitized using a Lab Scan (Amersham Pharmacia Biotech, Uppsala, Sweden) and converted into electronic files which were then analyzed by Image Master 2D software (Amersham Pharmacia Biotech, Uppsala, Sweden). Gel calibration was carried out on the basis of internal standard.

2D Immunoblot Analysis

Western blot analysis of total cellular extract and nuclear fractions of Raji and K562 was carried out as described previously after separation by two dimensional SDS–PAGE. Fifty micrograms of protein from each total and nuclear fraction were separated by 2D-PAGE and transferred onto polyvinylidene difluoride membrane, in accordance with Towbin et al. Two anti-CD38 monoclonal antibodies (AT1, IB4) were used at a concentration of 5 μg/ml. Immunocomplex, obtained using a specific peroxidase-labeled secondary antibody, was revealed by ECL plus method (Amersham Biosciences).

Ecto-GDPR Cyclase Activity

The ecto-cyclase activity of CD38 in K562 and Raji was evaluated by incubating with NGD^+, a

NAD^+ analogue, which is converted to cyclic GDPR which, unlike cADPR, is a fluorescent and cell impermeant end-product which can be detected in cell supernatants as an increase in fluorescence. Briefly, isolated nuclei, saturated and intact cells were resuspended in NGT buffer (0.15 M NaCl, 5 mM glucose, 10 mM Tris-Cl, pH 7.4). To the cellular and nuclear suspensions 10 μl 10 mM NGD (Sigma) in 20 mM Tris, pH 7.4 or 10 μl buffer (control) were added. After 30 min at 37°C, supernatants were collected by brief centrifugation. To evaluate conversion of NGD^+ to the fluorescent end-product cGDPR, supernatants were analyzed by fluorescence spectrometer (Perkin-Elmer, Boston, MA) set at excitation 310 nm and emission at 400 nm.

Intracellular Ca^{2+} Concentration

Intracellular Ca^{2+} concentration was measured in isolated nuclei and whole K562 and Raji cells using the fluorescent probe FURA 2-AM as previously described [Grynkiewicz et al., 1985]. Verifications were performed using a Perkin–Elmer LS 50 B spectrofluorometer at 37°C according to the method of Rao [1988]. Fluorescence intensity was evaluated at a constant emission wavelength (490 nm) with changes in the excitation wavelength (340 and 380 nm). At the end of any experiment, maximum and minimum fluorescence values, at each excitation wavelength, were obtained by first lyzing the cells with 0.1% Triton X-100 (maximum) and then adding 10 mM EGTA (minimum). With the maximum and minimum values, the 340/380 nm fluorescence ratios were converted into free Ca^{2+} concentrations using a FURA-2 Ca^{2+} binding constant (135 nM) and the formula described by Grynkiewicz et al. [1985].

Chemicals and Materials

All reagents and tissue-culture media used were obtained from Sigma (Milan, Italy), unless otherwise indicated. The chemical cross-linkers disuccinimidylsuberate (DSS) and dithio-bis-succinimidylpropionate (DSP) were obtained from Pierce (Rockford, IL). The Nonidet P-40 and Triton X-100 detergents were obtained from Fluka (Buchs, Switzerland). Carrier-free $Na^{125}I$, nitrocellulose (Hybond-C extra), and reagents for Western blotting and chemiluminescent detection of mouse and rabbit antibodies were purchased from Amersham (Milan, Italy). The silver-staining kit and prestained SDS/PAGE standards were purchased from Bio-Rad, *Staphylococcus aureus* protease-V8 from Miles Laboratories (Naperville, IL) and Centricon-10 from Amicon (Milan, Italy). The following mAbs, a kind gift from F. Malavasi, were used: HPLC affinity-purified murine anti-CD38 IB4 (IgG_{2a}) and AT1 (IgG1) clones. In this research, we have repeated our experiments changing the primary anti-CD38 monoclonal antibody to exclude a non-specific binding; the choice of the two antibodies used in combination was based on data from previous works [Hoshino et al., 1997; Ferrero et al., 2004] which have divided the currently existing antibodies into two subsets, according to the recognized epitope of CD38. IB4 and AT1 belong to different subsets and recognized diverse CD38 epitope; their concomitant use guarantees that the signal is the result of a specific recognition and binding to CD38. Anti-β-tubulin antibody was from Sigma (T4026, IgG1); anti p80-coilin rabbit polyclonal IgG (sc-32860) and the goat anti-rabbit IgG-TR (sc-2780) secondary antibody were from Santa Cruz Biotechnology. Immobiline DryStrip (3–10 NL) and other electrophoretic reagents were from Amersham Pharmacia Biotech (Uppsala, Sweden). 3-3 (Cholamidolpropyl) Dimethylammonio-1-propane sulfonate (CHAPS), Tributyl phosphine (TBP), Acrylamide, piperazine diacrylamide (PDA), sodium dodecyl sulfate (SDS), ammonium persulfate (APS), TEMED, glycerol, bromophenol blue, Tris, glycine, ammonium bicarbonate, α-cyano-4-hydroxycin-namic acid (CHCA) and peptide calibrant for MALDI-TOF analysis were obtained from Sigma Chemical (St. Louis, MO). All other chemicals were of analytical reagent grade. All buffers were prepared with Milli-Q water system (Millipore, Bedford, MA).

The instrument for running the first–dimensional IPG gels (IPGphor) was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Casting and running second-dimensional SDS–PAGE (Mini Protean III) were purchased from Bio-Rad Laboratories (Hercules, CA).

RESULTS

Morphological Analysis

The investigations by light and electron microscopy have been performed to recognize the nuclear and subnuclear organization of Raji and K562 cells and the accuracy of nuclear

preparation along morphological and biochemical evaluations. Briefly, Raji cells at low magnification, as reported in Figure 1, section A, display the standard cellular and nuclear arrangement of human lymphoblastoid cells. Into the nuclei is often possible to observe one or more round-like dark-prestained bodies. These bodies are morphologically distinguishable from nucleoli. Higher magnification of intact Raji cells (Fig. 1, section B) details the data observed at low magnification. The cytoplasm of Raji cells appears abundant with the presence of many organelles such as mitochondria, RER profile and free ribosome. The plasma membrane is mostly smooth or with few microvillus or blebs. The electron microscopy analyses of Raji and K562 nuclear preparations (Fig. 1, sections C and D) give the possibility to delineate the fine nuclear structure, which appears mostly unaltered by extraction procedure. These preparations also prove the absence of cellular membrane contaminations. Highly purified nuclei, delimitated by inner membrane, maintain intact nuclear arrays with the presence of dark bodies as previously observed at low magnification. These bodies, due to their characteristic shape and size, are analogous to micronuclei or Cajal bodies. Cajal bodies are small nuclear organelles found in a variety of animal and plant nuclei, suggesting that they are involved in basic cellular processes [Gall, 2000; Platani and Lamond, 2004].

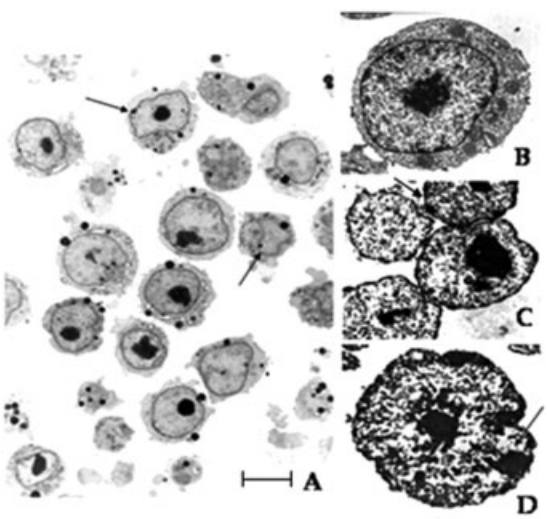

Fig. 1. Morphological features of Raji and K562 cells or nuclei observed at transmission electron microscopy level. Note the purity of nuclear preparation. Arrows indicate the presence of nuclear bodies both in intact cells or isolated nuclei. **A**: Semithin section of Raji cells counterstained by toluidine blue. **B**,**C**: Higher magnification of Raji cells and nuclei respectively. **D**: Isolated nucleus from K562 cells. Bars (A) 15 μm; (B) 6 μm; (C) 2 μm; (D) 3 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

CLSM Analyses and CD38 Localization

Figure 2 reports the confocal immunolocalization of CD38 in K562 (sections A, B) and in Raji (sections C, D) cell lines. In agreement with other data, Raji cells (section C) express on their cell surface CD38 while K562 cells (section A) do not express this antigen. In this section is possible also to detect a fine green staining outside the nucleus, due to the presence of CD38 at cytoplasmic level. This observation is in agreement with other previous works [Khoo and Chang, 1999; Deaglio et al., 2001a] and may be correlated to the site of CD38 synthesis. Nuclear preparations (sections B, D) prove the presence of CD38 at nuclear level in both cell lines. Comparing the images from PI red staining, which is able to clarify the DNA arrangement and then the nuclear cyto-architecture, with those from FITC-labeled CD38 antibody which green stains the immunocomplex sites, is possible to detail the fine distribution of CD38. Merging images explain that CD38 FITC-labeled antibody carefully dyes all nucleus. In particular nuclear regions containing less amount of condensed red chromatin are mostly green stained. Moreover one or more bright green areas within the nucleus are evident; they correspond to a nearly circular formation seen under electron microscopy conditions. Section D reports a confocal gallery of Raji nuclear preparation. These images which show different nuclear sections prove that CD38 is finely distributed within all the nucleus. These sections moreover confirm the presence of round FITC positive bodies.

To authenticate the specificity of the immunofluorescence procedures several controls have been performed. At the first we used two different monoclonal antibodies anti-CD38, AT1 and IB4 clones. The analyses of nuclear preparation derived from both cell lines basically give the same results using indifferently AT1 or IB4 (Fig. 3). All purified nuclei are CD38 positive independently of the antibody used, evidencing a significant presence of this protein. The second control has been carried out using as primary antibody a monoclonal recognizing HLA class I molecule, present only at cell surface compartment. Figure 4, reporting these nuclear preparations does not show the

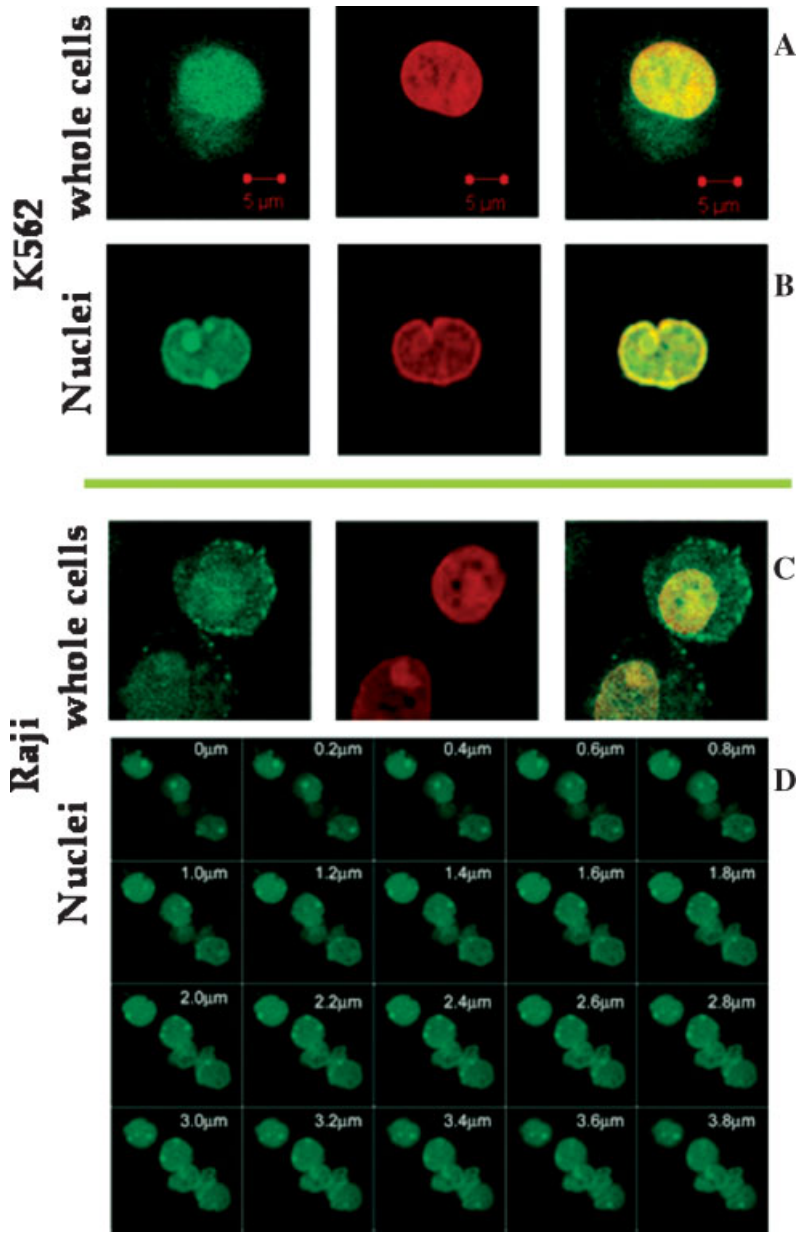

Fig. 2. Panel **A**–**C**: Immunolocalization of CD38 by FACS analysis in K562 and Raji samples. The pictures show the green stained FITC immunocomplexes, PI red-stained chromatin and electronically merged-pseudo staining. Immunophenotipically Raji cells are CD38 positive while K562 are, as expected, negative, even if they show the presence of CD38 at cytoplasmic level. CD38 is homogeneously distributed within the PI unstained nuclear structure. Some roundish highly FITC-stained bodies are present. Panel D: Reports gallery images of nuclear preparation demonstrating the fine distribution of CD38.

presence of positive HLA immunocomplexes. Therefore, confocal analyses display that the homogeneous scaffold shape-like distribution of CD38 appears similar in permeabilized cells or in purified nuclei of Raji and K562 samples. Several cells and nuclei exhibit a green-bright round-like structure comparable to micronucleoli or to Cajal bodies (also known as coiled bodies, or Cbs). Cajal bodies are subnuclear organelles that contain specific nuclear antigens, including splicing small nuclear ribonucleoproteins (snRNPs) and a subset of nucleolar proteins; they are localized in the nucleoplasm, often found at the nucleolar periphery, and appear like mobile structures, moving, fusing, and budding within the nucleus. To establish the precise localization of CD38 in connection to these bodies, we performed a

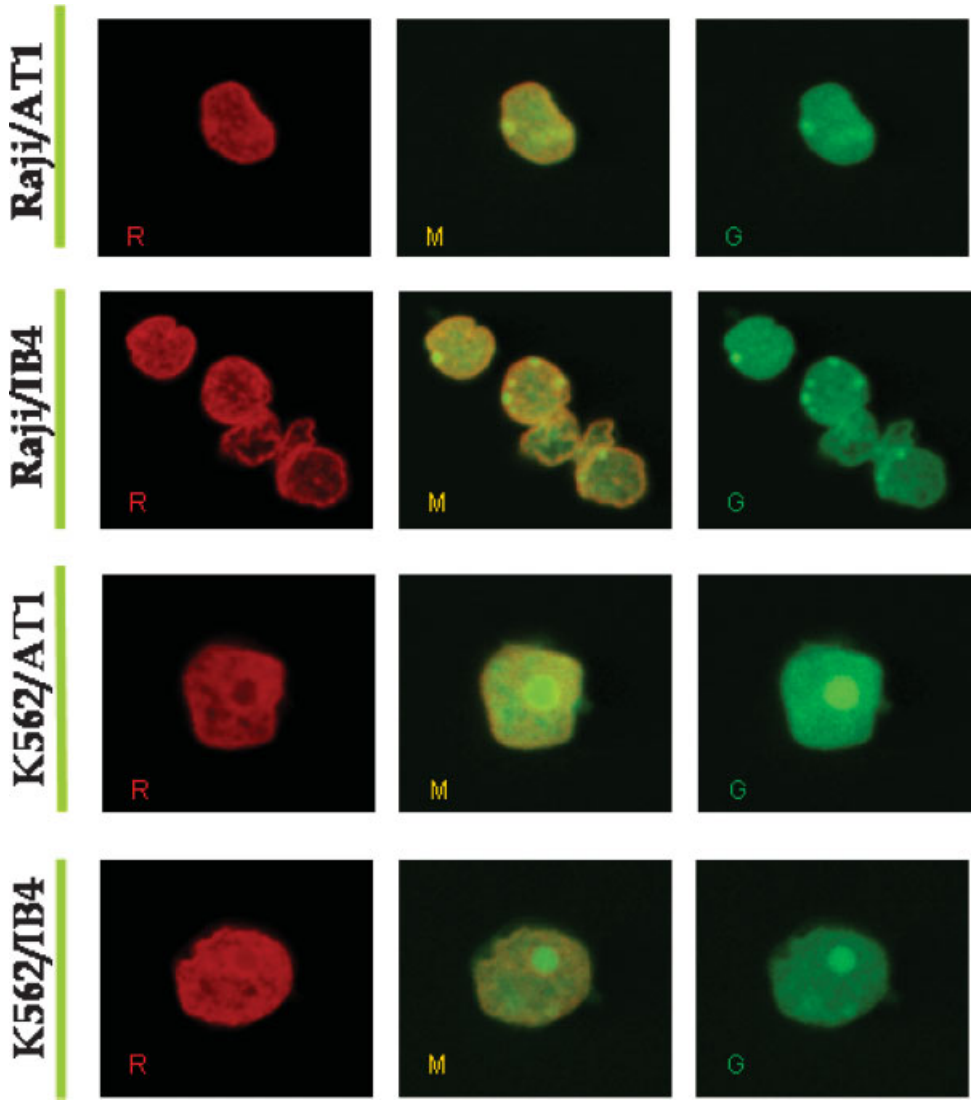

Fig. 3. FACS analysis of Raji and K562 nuclear preparations treated with two different CD38 monoclonal antibodies, IB4 and AT1. The stainings coincide, proving the presence of CD38 within the nucleus. Nuclei contain FITC prestained bodies which localize in PI red-unstained regions.

double immunostaining of Raji and K562 nuclei with antibodies recognizing CD38 and p80-coilin antigens. As reported in Figure 4, confocal laser scanning microscopy analysis proves the localization of both these antigens in nuclear preparation. Moreover, as reported in the insert and in the graph, displaying the intensity profile along the dotted line traced over the p80-coilin spot, we can observe the p80-coilin and CD38 co-localization in Cajal body.

To confirm the nuclear localization of CD38, an immunoelectron microscope study has been performed. Using immunogold technique, it has been possible to analyze the location of CD38 in situ cells, and as shown by confocal determination, both cell lines appear to contain molecules of CD38 at nuclear level. Figure 5 section A reports a higher magnification of Raji nucleus proving that CD38 is confined to interchromatinic regions. It is possible for the first time to establish that CD38 within the nuclei is arranged in a particular nuclear domain.

The nuclear presence of CD38 has been confirmed by biochemical studies. In particular we focused our attention on proteomic determination of CD38 molecule both in intact cells and purified nuclei derived from Raji and K562 lines. Figure 5 section B reports the CD38 immune precipitate of nuclear extracts from Raji and K562 cells. The specific 42–45 kDa

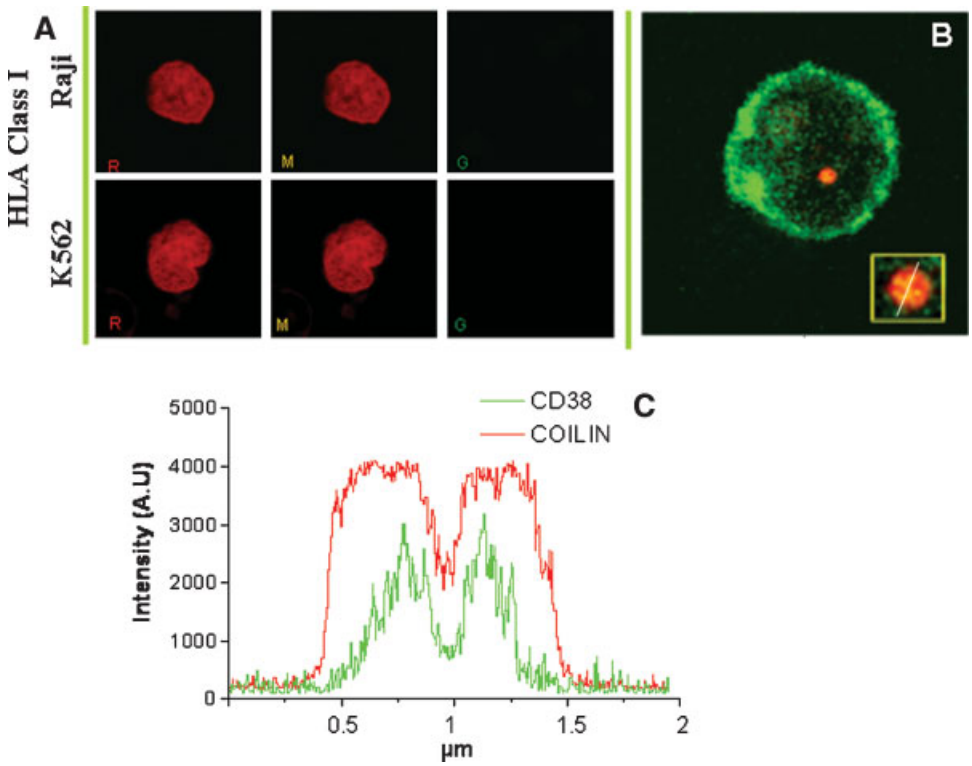

Fig. 4. **A**: Control of immunostaining procedure using anti-HLA I monoclonal antibody. As expected, no green positive immunocomplexes are present. R, PI staining; M, merged; G, FITC staining. **B**: Confocal demonstration of CD38 and p80-colinin localization in Raji nuclear preparation obtained as previously described. The insert shows a higher magnification of Cajal body while the graph (**C**) reports the intensity profile along the dotted line (white bar in the insert) traced over the p80-coilin spot displaying the p80-coilin and CD38 co-localization. Green: FITC-CD38; Red: Texas red p80-colin. Bar in the insert 2 μm.

band associated to CD38 is present in both samples. To evaluate the nuclear purification we used, as marker, the β-tubulin expression (Fig. 5, section C). Our results, as expected, show that the β-tubulin is detectable in both homogenates of Raji and K562 cells, while nuclei are negative.

Two-Dimensional Electrophoresis and Immunoblotting

We have used two-dimensional gel electrophoresis in our study to obtain the protein profile of Raji and K562 cells and nuclei as reported in Figure 6. The data obtained from gel electrophoresis prove a detectable presence of spots, with molecular weight equivalent to CD38.

Sections A and C, referred to total lysates, demonstrates a higher level of CD38 in Raji cells than in K562 cells due to the addition of transmembrane form while, in nuclear compartment (sections B–D) the amount of CD38 is comparable in both extracts. Moreover, because the levels of CD38 in 50 μg of total protein lysates is not proportional to that included in 50 μg of nuclear proteins, the nuclear spots can be compared each other but not with those in total lysates panels.

To confirm in an immunological way that the spots recovered by 2D gel electrophoresis are referred to CD38 we performed a Western blotting analysis.

At this purpose, loaded proteins were electrophoretically transferred to nitrocellulose membrane and then treated with IB4 antibody anti-CD38. The immunocomplexes were revealed by ECL plus method proving that, as expected, the spots in each panel (Fig. 7, A–D) are due to CD38 molecules confirming 2D electrophoresis observation.

It is important to note that the data reported in Figure 7 serve only as demonstration of the presence of CD38 in specific spots, but do not give any quantitative information since the method used to reveal immunocomplex, was suited for each panel to make an easier visualization of CD38. Moreover, K562 nuclear extract (section D) shows additional spots (arrow) at molecular weight ranging from

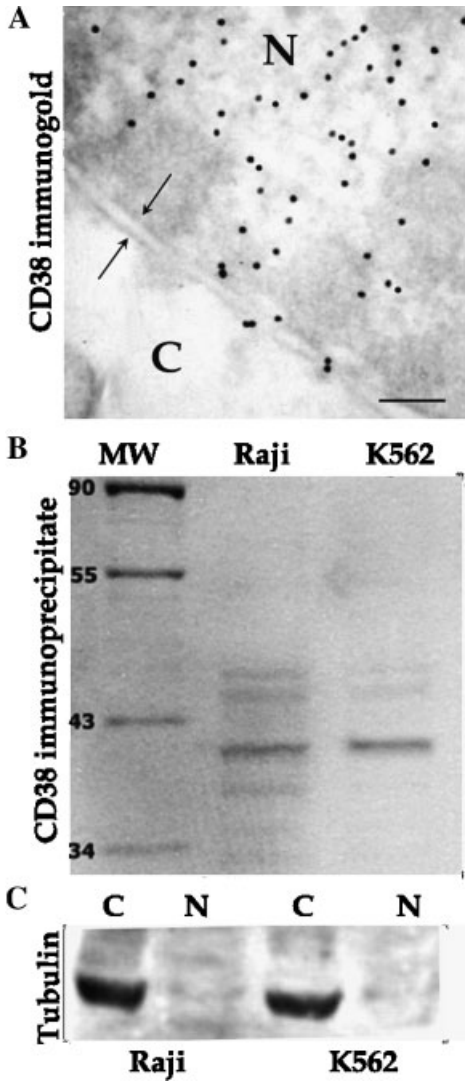

Fig. 5. **A**: Gold immunolocalization of CD38 in Raji cells performed as described in Materials and Methods. Gold particles are exclusively localized at interchromatinic regions finely distributed. N, nucleus; C, cytoplasm. Bar:1 μm. **B**: Immunoblotting of CD38 protein level obtained from Raji and K562 nuclei. MW: molecular weight. **C**: expression of β-tubulin in whole cells (C) and isolated nuclei (N) from Raji and K562. Nuclear preparations prove the absence of β-tubulin in this cellular compartment. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

35,000 to 33,400 Da and isoelectric point from 7.6 to 8.0. This finding may suggest the idea that CD38s detected in K562 nuclear extracts are subjected to post translational modifications. These results need to be confirmed by protein sequence analyses.

Ecto-GDPR Cyclase Activity

The ADP-ribosyl cyclase activity was measured fluorometrically as the production of cGDPR from the substrate NGD. As reported in Figure 8 section A, the cyclase activity of K562 permeabilized cells coincides with that detected in isolated nuclei, while no activity was found in intact cells. These results are in agreement with the presence of CD38 only in the nucleus. In Raji cells, instead, the observed higher value of fluorescence in permeabilized whole cells is due to the amount of nuclear and transmembrane CD38 cyclase-activity. It is important to note that the level of nuclear enzymatic activity is almost the same for Raji and K562 cells, confirming that the nuclear CD38 expression is independent from that of the transmembrane.

Intracellular Ca^{2+} Concentration

After demonstrating the cyclase activity of CD38, we examined the intracellular Ca^{2+} concentration in isolated nuclei and whole K562 and Raji cells using the fluorescent probe FURA 2-AM (Fig. 8 section B). As expected after the analysis of cyclase activity, Ca^{2+} level is more elevated in Raji than in K562 cells (316.82 nM vs. 187.3 nM), while in isolated nuclei the values of Ca^{2+} concentration are 17.25 and 14.08 nM in Raji and K562 cells respectively. The data of cyclase activity and intracellular Ca^{2+} concentration are connected to each other and reflect the expression of CD38. The molecule in fact is able to catalyze the production of cyclic adenosine diphosphate ribose (cADPR) that is involved in calcium mobilization.

DISCUSSION

Protein localization is a highly dynamic biological process. To ensure a proper cellular function, the spatial distribution of different proteins needs to be delicately regulated and coordinated.

CD38 is a lymphocyte surface antigen originally defined by monoclonal antibody typing [Reinherz et al., 1980]. Its expression in lymphocytes is stage-specific and has been widely used as a marker for lymphocyte differentiation [Malavasi et al., 1992]. 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 [Deaglio et al., 2001b].

CD38 is a multifunctional enzyme ubiquitously distributed in mammalian tissues working as a bifunctional ectoenzyme catalyzing the synthesis and hydrolysis of cyclic ADP-ribose (cADPR).

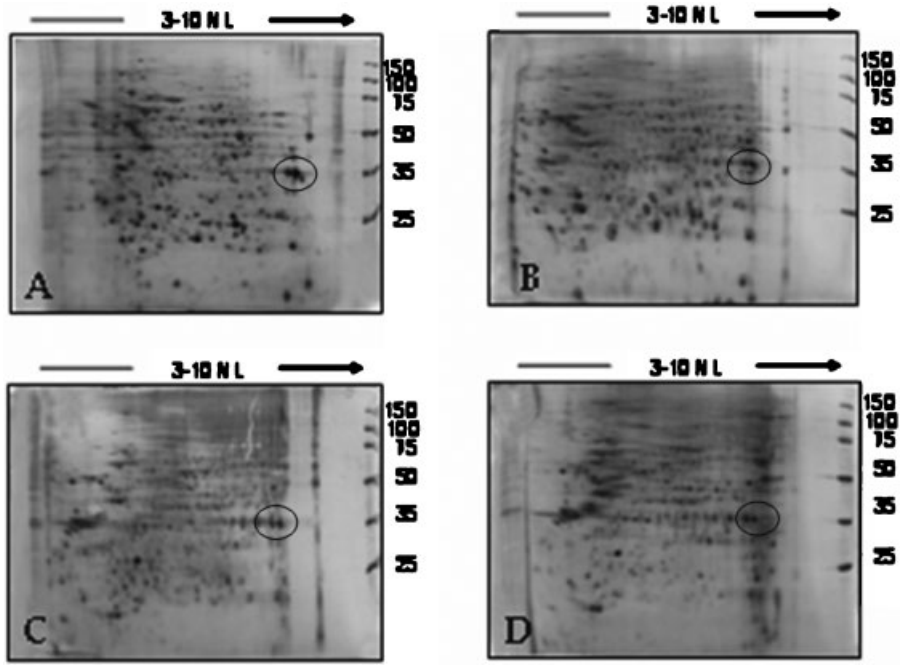

Fig. 6. Two dimensional gel. **A**: Raji total extracts; (**B**) Raji nuclear extracts; (**C**) K562 total extracts; (**D**) K562 nuclear extracts. CD38 protein spots are indicated with circles. Y-axis reports MW marker. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

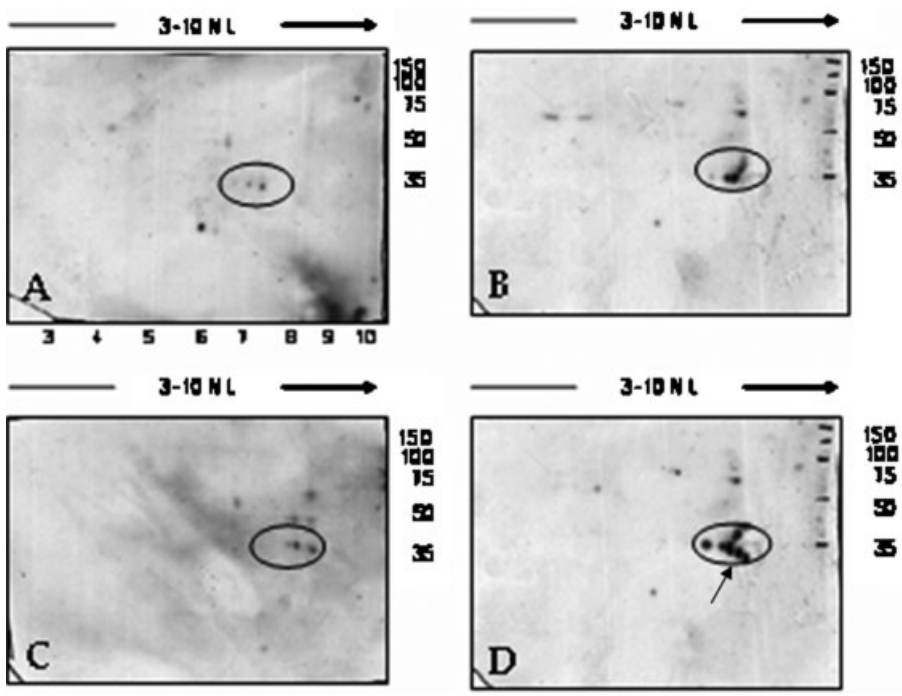

Fig. 7. CD38 Western blotting analysis. **A**: Raji total extracts; (**B**) Raji nuclear extracts; (**C**) K562 total extracts; (**D**) K562 nuclear extracts. CD38 immunoprecipitate are indicated with circles. Nuclear preparation of K562 displays other isoforms (arrow) with an isoelectric point (pI) ranged from 7.6 to 8.0 and molecular weight around 35 kDa. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

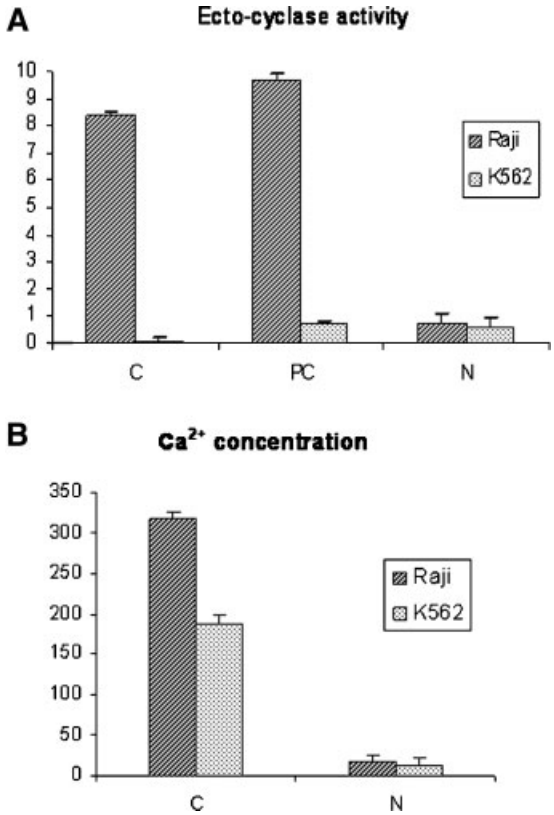

Fig. 8. **A**: Detection of ADP-ribosyl cyclase activity. The ADP-ribosyl cyclase activity was determined by measuring the formation of the fluorescence as the production of cGDPR from the substrate NGD^+. The indicated fluorescences have been calculated like the difference between the values observed in presence and in absence of NGD^+ for each preparation (C, whole cells; PC, permeabilized cells; N, isolated nuclei). Results shown are representative of three separate experiments. Y-axis = fluor-fluorescence emission 410 nm. **B**: Intracellular Ca^{2+} concentration. The measuring of intracellular Ca^{2+} was determined by the fluorescent probe FURA 2-AM in K562 and Raji whole cells (C) and isolated nuclei (N). Fluorescence intensity was evaluated at a constant emission wavelength (490 nm) with changes in the excitation wavelength (340 and 380 nm). Results shown are representative of three separate experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

cADPR is a universal second messenger that releases calcium from intracellular stores. However, major enzymatic activity of CD38 is the hydrolysis of NAD^+, in fact, CD38 will generate one molecule of cADPR for every 100 molecules of NAD^+ hydrolyzed. Akasoy et al. postulated that CD38 is the major NADase in mammalian cells and that it regulates intracellular NAD^+ levels. To date, the role of CD38 as a modulator of levels of NAD^+ has not been explored.

CD38 has been shown to be present on the surface of many cell types and, although CD38 is known essentially to be an ectoenzyme found on the plasma membrane, previous reports have indicated the possible existence of CD38 in other spatial sites including a soluble form [Funaro et al., 1996] as well as an intracellular site particularly in the nuclear envelope [Khoo and Chang, 1998]. Furthermore, Khoo and Chang showed that CD38 is not detected in the nuclear membranes of permeabilized B cells and that CD38-dependent enzyme activity is absent in B cell nuclear pellets and nuclear lysates. These data may indicate that CD38 is not localized in the nuclear membranes of mature B lymphocytes. Even though these results are in contrast to previous reports showing the presence of CD38 in the nucleus of several cell types [Khoo and Chang, 1998, 1999, 2002; Adebanjo et al., 1999; Khoo et al., 2000; Ceni et al., 2002]. Khoo and Chang suggest that they may not be contradictory. In fact, in almost all cases the cells examined were isolated from non-hematopoietic tissues like the brain, liver and osteoclasts, where alternative forms of CD38 may be expressed. Only one group reported that CD38 is expressed in the nucleus of rat lymphocytes and this report did not show the purity of their nuclear fraction [Khoo and Chang, 2002], and it may be possible that this nuclear fraction was slightly contaminated with plasma membrane proteins. In consequence of this the divergence on CD38 nuclear presence may simply reflect species differences or differences between how the nuclear fractions were isolated. The results described in this study provide a possible resolution to this topological issue. Here we show that CD38 can also be an intranuclear active enzyme. Its localization within the nucleus in CD38 positive and negative cells represents the first demonstration of selective association of CD38 with the nuclear compartment. Moreover, our study does not allow us to reach a definite conclusion with regard to the precise function of the CD38 within the nucleus but suggests that the model of CD38, being essentially an enzyme found mainly on the plasma membrane, has to be modified since it appears that CD38 has various subnuclear spatial localization sites. These results indicate that the presence of CD38 in the nuclear environment might have functional and regulatory roles in the nucleus since it retains Ca^{2+} mobilizing cADPR-dependent activity.

The cell nucleus is a complex and highly dynamic environment with many functionally

specialized regions of substructures that form and maintain themselves in the absence of membranes. Control of the nuclear localization of specific proteins represents an important mechanism for regulating many signal transduction pathways. The majority of these proteins, including novel proteins and proteins initially categorized as cytoplasmic proteins, were found to be also localized in the nucleus. Detailed characterization of these proteins revealed various subnuclear localizations, indicating their possible association with chromatin and the nuclear matrix with a foci or speckle-like distribution. The presence of CD38 at the nuclear level appears not to be a surprise since many other molecules [Conner and Schmid, 2003], previously classified as cytoplamic or membrane linked, have now been recovered within the nucleus. This is not without precedence also in signal transduction. For example, multiple IP_3 receptor isoforms have been shown to be present both on the plasma membrane and internal membranes [Quinton and Dean, 1996]. In addition, calcium stores mobilized by cADPR and NAADP have been shown to be co-purified with the ones sensitive to IP_3. Ca^{2+} metabolism, second messengers and nuclear localization have a good target represented from phosphoinositide pathway. Strong evidences prove that phosphoinositides, which are involved in the regulation of a large variety of cellular processes in the cytoplasm and in the plasma membrane, are present within the nucleus. Remarkably, the nuclear inositide metabolism is regulated independently from that present elsewhere in the cell [Cocco et al., 2006a,b] suggesting that there is also this possibility for the nuclear CD38. However, extensive studies on cytoplasmic-nucleo shuttling for a large number of cellular proteins [Macara, 2001], the specific mechanism on how a cellular protein travels through the nuclear membrane component other than NPC still remains unsolved.

More systemic studies are required to clearly define the detailed mechanism of CD38 nuclear translocation since the control of the nuclear localization of specific proteins represents an important mechanism for regulating many signal transduction pathways.

Compared with the compartmentalization of the cytoplasm, the nucleus has long been considered as relatively unstructured. In the absence of membrane-delimited organelles, the nuclear organization is based on functional compartments, or domains, whose spatial localization involves the nuclear matrix, which shares many properties with the cytoskeleton [Cocco et al., 2004]. The nuclear domains are not necessarily stable but dynamically variable, which perform specific metabolic functions through the partitioning of molecular complexes which appear involved in several diseases [Maraldi et al., 2003, 2005]. As described confocal analyses display that the homogeneous nuclear scaffold shape-like distribution of CD38 appears equivalent in saturated cells or in purified nuclei of Raji and K562 preparation. Moreover several nuclei display CD38 green-bright round-like structures corresponding to micronucleoli or to Cajal bodies. By experimenting with immunostaining we prove that CD38 co localizes with p80-coilin which is the marker of these nuclear bodies.

Cajal bodies function is still in part elusive, recent works suggest that they are involved in several nuclear functions, including modification of small nuclear RNAs and small nuclear ribonucleoproteins, important for spliceosome formation, and assembly of the three eukaryotic RNA polymerases (pol I, pol II, and pol III) with their respective transcription and processing factors that are then transported as multiprotein complexes to the sites of transcription. Interestingly, Dundr et al. [2004] suggest the existence of compartment-specific retention mechanisms for proteins in CBs and nucleoli, and our confocal data are in agreement with these results suggesting that CBs could have the role of nuclear CD38 store.

Our findings demonstrate that nuclear CD38 exists in a non-membranous form, linked to structural nuclear proteins of the inner nuclear matrix. They also suggest that the CD38 in the nucleus is completely independent of that of the cell surface and that it probably, as previously suggested, [Martelli et al., 2005] preceded in evolution the systems which are present at the cytoskeletal and cell membrane level.

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