DP58, an inducible myeloid protein, is constitutively expressed in murine neuronal nuclei

Nisreen Alshaibi[1], Michael W. King[2], Taihung Duong[3], Swapan K. Ghosh[1]

[1]Department of Life Sciences, Indiana State University, Terre Haute, IN 47809, [2]Center for Regenerative Biology and Medicine, and [3]Anatomy and Cell Biology, Indiana University School of Medicine, Terre Haute, IN 47809

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1. ABSTRACT

A novel cytosolic phosphoprotein, DP58 induced in bone marrow-derived dendritic progenitors was found in this study to be constitutively expressed at a very high level in neuronal nuclei. Amplified cDNA confirmed by sequencing to be DP58 was present only in brain tissue, and DP58-like protein was expressed in neurons as a 52 kDa nuclear protein, phosphorylated primarily at the serine residues. In contrast, its isoform in dendritic progenitors appeared as a 58 kDa inducible protein with phosphorylation at serine, threonine and tyrosine residues. Although protein markers common to brain and hematopoietic cells are known, no report was found on constitutive expression in neuronal nuclei of DP58, an inducible Pro-myloid marker. The sequence of DP58 reveals ankyrin repeats present in a wide spectrum of interacting proteins including NF-kappaB-binding BCL3, a predominantly nuclear protein of I-kappaB family. The contrasting phosphorylated forms of DP58 suggest a distinct physiological role in neuronal cells and early dendritic progenitors.

2. INTRODUCTION

The development of bone marrow (BM)-derived hematopoietic cells are crucial against infections, and various lineages or descendants play distinct and vital roles in the vertebrate immune system. These developmental processes are accompanied by expression or down regulation of many stage-specific phenotypic markers, which in turn shed light on various intermediary steps leading to mature blood cells. However, not all biochemical events or differentiation markers associated with the generation of hematopoietic cells, particularly at the progenitor levels, are fully known (1-2). In addition, some phenotypic markers may even be shared between two groups of cells that originate from embryologically different germ layers. However shared phenotypic markers may be useful to explore transdifferentiation capability, and the potentials for cross-talk and co-localization in a specific physiological setting. Indeed, BM cells have been shown to change during the course of development and differentiate into endothelial cells, hepatocytes, muscle cells, cardiomyocytes and neurons (3-6).

Over the years, a number of phenotypic markers or antigens of BM cells have been shown to occur in non-immune cells, such as brain or neuronal cells (7-11). Many of these markers are associated with mature hematopoietic cells, but some appear at early stages of development. CD40 protein is expressed primarily in antigen-presenting mature cells such as B-cells, dendritic cells and monocytes/macrophages. It also occurs constitutively on neuronal cells (12). Conversely, hematopoietic progenitors can express neurogenic transcription factor Pax-6 and neuronal nuclear protein NeuN (12). This is not surprising since there are many examples of phenotypic and functional similarities between brain and bone marrow cells. The functional similarities extend from their ability to form synapses for cell-cell communication to information processing and generation of short and long-term memory. Besides, both systems are distributed throughout the body, and they influence each other by phenotypic interactions mediated by characteristics products such as cytokines and neurotransmitters (13-15).

We previously identified and described a novel cytosolic phosphoprotein DP58 induced in early progenitor dendritic cells (pro-DCs) of myloid lineage (16). DP58 in BM-derived hematopoietic stem cells is identical in sequence to a protein corresponding to a computationally predicted Riken cDNA (8). Because it is undetectable when Pro-DCs develop into immature and mature DCs, DP58 is a phenotypic marker of Pro-DCs. As a first step to understand the physiological roles of DP58 in the context of BM differentiation, we evaluated the tissue-specificity of DP58, its cellular localization and its phosphorylation status. Using biochemical and molecular techniques we show that, unlike in BM, DP58 is constitutively expressed in brain, although protein sequences in both tissues are completely identical. Another interesting difference lies in their phosphorylation forms in brain and differentiating BM cells. Whereas the BM4 cell (Pro-DC)-derived DP58 is phosphorylated in serine, threonine and tyrosine residues, its counterpart in brain tissues shows phosphorylation primarily at serine residues. Moreover, immunocytochemical studies demonstrate copious presence of DP58 in the cell nuclei of cultured neurons as well as in neurons of the basal ganglia, brainstem and neocortex of adult mice brain tissues. We are currently exploring the possibility that this novel phosphoprotein may function in a manner similar to BCL3-like nuclear proteins in specific tissues. Furthermore, because of its tissue-specific expression in brain and BM cells, DP58 isoforms may prove useful for monitoring hematopoietic stem cell differentiation into neuronal cells (Patent pending).

3. MATERIALS AND METHODS

3.1. Animals

BALB/c mice from Harland Sprague-Dawley (Indianapolis, IN) were bred in the animal facility of Indiana State University. The University Animal Care and Use Committee (ACUC) approved all animal experiments described in this article.

3.2. Antibody reagents

For phenotypic characterization of immature DCs and BM progenitors, we used the following monoclonal antibodies conjugated to FITC, and directed to MHC class II, CD11b, B220, CD86, CD11c, CD8α, CD80 and CD117(all from eBioscience, USA). Antibodies to phosphotyrosine, phosphoserine and phosphothreonine were obtained from Zymed, USA. These were used in Western blotting to determine the phosphorylation status of DP58 in Pro-DCs and brain cells. Some reagents for Western blotting and immunocytochemistry were purchased from Pierce (USA), anti-rabbit-HRP (ICN, USA) goat anti-rabbit Cy3, and goat anti-mouse Cy2, (Amersham, USA). We purchased monoclonal anti-acetylated tubulin (clone 6-11B) from Sigma Chemical, USA, and the dye, 4, 6 diamidino-2-phenylindole, dilactate (DAPI, dilactate) from Invitrogen, USA.

3.3. Generation of Dendritic and promyloid Dendritic progenitor cells

This has been performed as described in a previous publication (16). Briefly, single-cell suspensions from bone marrow were prepared by isolating cells from femurs and tibia of mice. BM cells were cultivated in Iscove-modified Dulbeccos medium (IMDM) containing10ng/ml recombinant granulocyte macrophage colony stimulating factor (GM-CSF from eBioscience and peprotech, USA) and 10% FBS for 6 days in 5% CO_2. Non-adherent cells were removed from the culture on days 2 and 4 and fresh IMDM supplemented with GM-CSF was added to the culture dish. Immature DCs (IDCs) were generated in 6 days. Fluorescent microscopy and flow cytometry were used to identify DCs.

The promyloid DCs termed BM4 cells were generated by incubating freshly harvested BM cells for 4 hour on ice with a rabbit anti-DC differentiating antibody preparation described in our earlier publication (16).

3.4. Anti- DP58 peptide antibody reagent

We previously described identification, purification and sequencing of a cytosolic phosphoprotein DP58 from the lysates of BM4 cells, a promyloid DC progenitor population (16). A DP58-specific peptide, KMVKYLLENSADPNQDKSG-conjugated to keyhole limpet hemocyanin (KLH) was used to raise rabbit antisera which were purified by sequential adsorption and affinity-purification (16). The DP58 peptide-specific rabbit antibody reagent was used in Western blots to detect DP58-like proteins in BM, BM4, immature DCs and mouse brain tissues. The purified anti-DP58 antibody was found by enzyme-linked immunosorbent assays to be uniquely specific for the above peptide only.

3.5. Isolation of nuclear and cytosolic fractions to detect DP58 protein

The isolation from BM4 cells and brain tissues was carried out according to a published procedure (17). Briefly, murine brain tissues were homogenized using a Dounce homogenizer in a medium containing 0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$ and 20 mM Hepes-KOH. The brain homogenates were filtered though 4 layers of cheesecloth. BM4 cells were also prepared in the same medium using an ultrasonic converter. The homogenates of brain tissues or BM4 cells were then centrifuged at

1000g for 10 min to sediment the nuclear fractions. The supernatant fraction was further centrifuged at 17000g for 10 min to obtain the post-nuclear cytosolic fractions. Both nuclei and cytosolic fractions were then resuspended separately in 1 ml of a lysis buffer, described before (16). The resulting lysates dissolved in SDS sample buffer were subjected to SDS-PAGE, as described (16). Electrophoretically separated proteins were then transferred onto nitrocellulose membranes for Western blotting (16). Rabbit anti-DP58 peptide was used as the primary antibody, followed by goat anti-rabbit-Ig-HRP, and Super Signal West Pico chemiluminescent substrate for visualization of the band

3.6. Immunoprecipitation and Western blotting

Following a published procedure (18), the lysates of brain, BM4 and undifferentiated BM cells were immunoprecipitated using affinity-purified anti-DP58 antibody. First, the anti-DP58 antibody was incubated with beads of protein A-agarose for 2 hours at 4° C; beads were washed in the extraction buffer (containing 0.5% MEGA9, 150 mM NaCl, 5 mM EDTA , 50 mM Tris, 2 mM PMSF, 5 mM iodoacetamide) and blocked with 10% BSA. Then the cell lysates were incubated with the Protein A beads for 3 hours. The washed beads were suspended in the SDS sample buffer, boiled for 5 min and subjected to SDS-PAGE as described (18). Further confirmation of the identity of the bands separated by SDS-PAGE was done by Western blotting as described above using anti-DP58 as the primary antibody. Phosphorylation status of isolated DP58 protein was determined by Western blotting Commercial anti-phosphoserine, and anti-phosphothreonine, and anti-phosphotyrosine antibodies were used as the primary antibodies on Western blots.

3.7. DP58 occurrence in different tissues

The presence of DP58 in various tissues was examined by screening multiple tissue-specific cDNAs (MTC Panels cat no. #K1441-1 and #K1430-1 from BD Biosciences Clontech, USA) by PCR. The MTC panels included cDNAs from normal mouse tissues such as heart, spleen, and lung, liver, skeletal muscle, kidney, testis, embryos of various ages, bone marrow, eye, lymph node, smooth muscle, prostate, thymus, uterus, and stomach. We also screened for DP58 by PCR, freshly isolated bone marrow cells, brain tissues, pro-DCs (BM4 cells), immature and mature DCs from mice of various ages and confirmed by sequencing of the amplified DNA band.

3.8. Reverse-transcriptase mediated polymerase-chain reaction (RT-PCR)

Analysis of the expression of DP58 was carried out using RT-PCR essentially as described (20). The forward primer used was 5'-ATTCTTCTGAGACGGACCTGACAC-3' and the reverse primer consisted of 5'-CGCGTTGGTTTTGTAGGCTATTTC-3'. Total RNA samples were extracted using the RNAqueous system (Ambion, USA.). Reverse transcription reactions were carried out using 1μg of total RNA purified from indicated sources. Each reaction consisted of 60μl of which 25μl was the RNA and water. The RNA was denatured for 3

min. at 70°C then chilled on ice and the remaining reagents were added such that the reaction contained 1X reverse transcriptase buffer, 1mM $MgCl_2$, 0.5mM all 4 dNTPs, 0.5 - 1μl RNase inhibitor (Promega, USA.), and 100pmole of random hexamers. The primers were allowed to anneal to the RNA at room temp for 5 –10 min. Lastly, 200U SuperscriptRT (Invitrogen, Inc.) was added and the reactions incubated for 60 min at 37°C. The RNA template was degraded by incubation with 1μg of RNaseA at 37°C for 15 min.

For end-point PCR reactions, an amount of the RT reaction equivalent to 16.7 ng of input RNA was subjected to the PCR. The reaction volume was 25 μl containing, 1X PCR buffer, 250 μM all 4 dNTPs, 2mM $MgCl_2$, 10 pmole of each specific PCR primer, and 2-3 units Taq polymerase. Reactions were standard 30 cycle PCRs with conditions involving an initial 5 min. 95C denaturation followed by 30-40 cycles of 95°C for 30 sec, 62°C for 30 sec, and 72°C for 30 sec. Following PCR, 10-15 μl of each reaction was analyzed by agarose gel electrophoresis and photographed by UV transillumination. As a control for RNA loading into the RT reaction, expression of *Mus musculus* glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was analyzed using a 25-cycle PCR. When expression was to be quantified by quantitative PCR (see below), the 60μl RT reaction was first diluted to 6-fold and 1μl of the diluted RT was used as template for each qPCR. The gel results of the RT-PCR data represent typical results obtained from at least three different assays with three different RNA isolates.

3.8.1. Quantitative PCR (qPCR)

Quantitative PCR was performed utilizing the Mx3000P PCR machine (Stratagene, USA). Fluorescence detection chemistry involved utilization of SYBR green dye master mix (Bio Rad, USA.). qPCR primers, the same as described above, were HPLC purified 24-mers composed of no more than 50% G-C content. Optimal primer concentrations were determined using a 5-fold reciprocal dilution series starting with a final primer concentration of 50 nM and ending with 250 nM. For quantitation, standard curves utilizing a 5-fold dilution series (from 25 ng to 0.04 ng RNA input equivalents) were performed for each primer pair. Each reaction consisted of optimal primer concentrations (generally found to be 150 nM for each primer), SYBR green master mix and an amount of RT reaction consisting of approximately 2.5 ng of RNA in the RT reaction. All qPCR reactions were carried out in triplicate and used a 40- cycle reaction whose time and temperature parameters were the same as for end-point PCR. Melting-curve analysis of all products demonstrated a single peak, indicating that each set of primers produced a single product. Each RT reaction was equalized for RNA input by assessing the level of expression of the relatively invariant housekeeping gene G3PDH. To determine quantitative values, standard curves were generated with each primer pair using a 5x dilution series ranging from 16.7 ng to 0.27 ng RNA equivalents of an RT. Expression of DP58 was then equated to the normalized input of G3PDH. The qPCR data are typical of results obtained from three independent assays.

3.9. Immunohistology and immunohistochemistry

Adult mice were euthanized with an overdose of sodium pentobarbital and transcardially perfused with neutral-buffered 4% paraformaldehyde. The brains were removed and post-fixed in the same fixative for 1 hour at room temperature on the shaker. They were then cryoprotected by immersion overnight in Tris-buffer (pH 7.4) with 30% sucrose. The brains were sectioned on a cryostat at a thickness of 40 µm. The sections were processed free-floating for immunohistochemistry as follows. All rinse steps were performed using Tris-buffer (pH 7.4). Sections were first incubated in methanolic peroxidase for 15 minutes to remove endogenous peroxidase and, following a rinse step, blocked with 5% non-fat dry milk for one hour at room temperature. They were then directly transferred to the primary antibody, DP58, diluted 1: 500 in 5% non-fat dry milk for 2 hours at room temperature and then overnight at 4°C; pre-immunization serum served as the negative control for the anti-DP58 antibody in all experiments. The next day, following a rinse step, the sections were incubated in peroxidase-conjugated goat anti-rabbit secondary antibody (diluted 1:100) for 3 hours at room temperature on the shaker. Following another rinse step, the immunolabeling was visualized by incubation with diaminobenzidine and hydrogen peroxide. The sections were mounted on alcohol-gelatinized slides and cover-slipped with Permount.

A similar procedure was used for immunohistofluorescence to visualize neurons and BM4 cells, with the following differences: no endogenous peroxidase step was performed; the blocker was 3% normal goat serum; the primary antibody was mixed in 1% normal goat serum; and the secondary antibody was Cy3-linked goat anti-rabbit IgG, diluted 1:1000. Sections were also double-labeled using DP58 antibody and a mouse antibody to microtubule-associated protein 2 (MAP2; Sigma product # M-1406; diluted 1:250) as a neuronal marker. The secondary antibodies were Cy3-linked goat anti-rabbit IgG and Cy2-linked goat anti-mouse IgG, both diluted to 1:1000. A similar procedure was used for method control except that the primary antibody was replaced with pre-immunization serum in each experiment.

3.9.1. Primary neuron culture

The primary neuron culture method was adapted from Brewer (19). Briefly, the hippocampus was isolated by dissection from mice brain, minced on a tissue chopper, incubated in Hibernate A (Gibco), and then treated with Papain (Worthington). The tissue was then triturated 10 times and the supernatant collected. The sediment was resuspended in Hibernate A/B27 (Gibco) and triturated again. This procedure was repeated once more with the supernatant saved each time. The collected supernatant was then layered on an Opti-Prep gradient and centrifuged for 15 minutes at 1900 rpm. The volume above the white suspension layer containing the neurons was discarded and the white suspension layer was transferred into Hibernate A. This was centrifuged at 1100 rpm at room temperature. The supernatant was discarded and the pellet resuspended in B27/Neurobasal A (Gibco). The neurons were plated at a density of 1 x 10^7 in Poly-D-Lysine-coated glass 96-well

culture plates containing B27/Neurobasal A. The cells were incubated for 1 hour at 37°C in 5% CO$_2$, rinsed with fresh B27/Neurobasal A at 37°C then in Hibernate A and incubated in growth medium. The cells were fed every other day and allowed to grow for 1 week before experiments were conducted. Visualization of nerve cells was performed using an antibody to the microtubule-associated protein 2 (MAP2), a component of nerve cell dendrites.

4. RESULTS

4.1. DP58 expression in different tissues

Expression of DP58 protein in various tissues was assessed by screening PCR multiple tissue-specific cDNAs (MTC Panels) using DP58-specific primers. The results in Figure 1A indicate that only the cDNA from brain tissues could be amplified using DP58-specific primers. Further corroboration of this finding came from RT-PCR and qPCR analyses of DP58 expression in freshly isolated whole brain, bone marrow cells, and cells generated during BM differentiation (Figure 1B). DP58 expression at the mRNA level in unstimulated brain far exceeded the levels seen in BM cells even after 40 cycles of PCR. The expression of DP58 message in brain and bone marrow was quantified by qPCR (Figure 1B). Brain tissue expressed approximately1200 times more DP58 mRNA than BM cells, particularly in dendritic progenitor cells BM4, when all DP58 levels were normalized with respect to the housekeeping gene G3PDH (Figure 1B). However at the protein level, DP58 expression was evident in BM4 but not in naïve BM or DC cells (Figure 1C), and in brain tissues only an isoform of this protein, namely DP52, was detectable. Interestingly, the latter did not express proportionately to what would be expected from mRNA shown in Figure 1B. To ensure that all lanes in Figure 1C were loaded equally with the same amount of protein of different tissues, we determined tubulin content in each lane using anti-tubulin monoclonal antibody (Figure 1C). Our results clearly indicated that unlike DCs, and unstimulated fresh BM cells, brain tissues constitutively expressed DP58 protein.

4.2. DP58 protein expression in brain and bone marrow cells

The demonstration that levels of DP58 mRNA level were higher in brain than in bone marrow cells raised the question of whether similar relative concentrations would be observed at the protein level in both tissues. By SDS-PAGE and Western blotting (Figure 1C), we show that contrary to what was seen with RT-PCR, DP58 protein is much higher in BM4 cells than in brain. Also, the estimated molecular weights were different. In brain tissue, DP58 migrated with an apparent MW of 52 kDa. Even though only Pro-DC or BM4 cells express DP58 protein, both BM4 and IDCs expressed similar levels of DP58 mRNA. Furthermore, in spite of several-fold high DP58 mRNA expression in unstimulated whole mouse brain tissue, it did not translate into proportionally high DP58 protein levels.
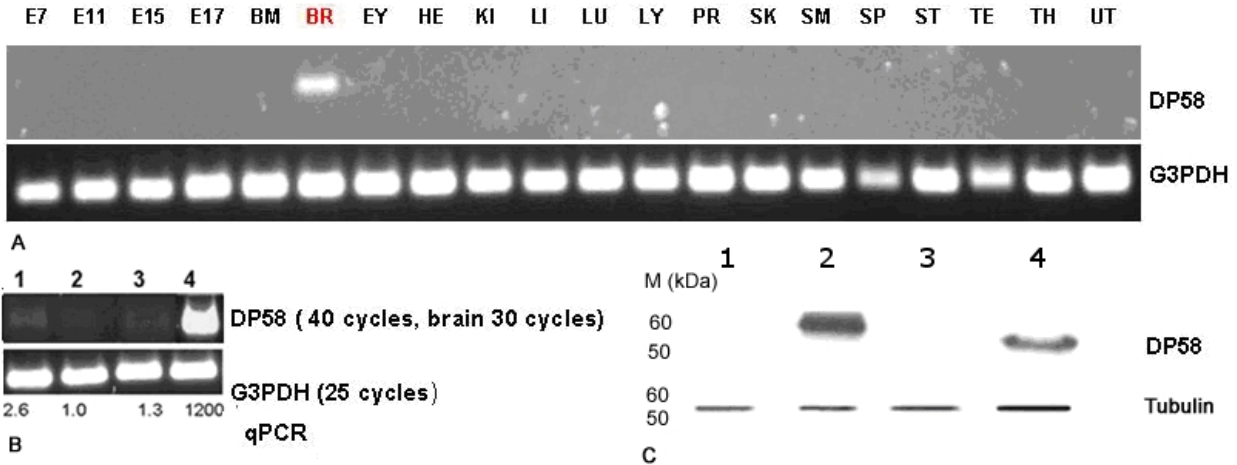
Figure 1. A. Analysis of DP58 expression in different mouse tissues using Clontech MTC panels (Clontech, Inc.) and comparison with expression of the housekeeping gene, gyceraldehyde 3-phosphate dehydrogenase (G3PDH). E7, E11, E15, E17 = embryonic days. BM = bone marrow; BR = brain; EY = eye; HE = heart; KI = kidney; LI = liver; LU = lung; LY = lymph; PR = prostate; SK = skeletal muscle; SM = smooth muscle; SP = spleen; ST = stomach; TE = testis; TH = thymus; UT = uterus. B. Analysis of DP58 expression during dendritic cell differentiation and in whole brain tissues using RT-PCR and qPCR. Lane 1: lysates of fresh bone marrow cells; lane 2: lysates of BM4 cells (bone marrow cells exposed for 4 hrs to anti-DC reagent); lane 3: lysates of immature DCs (IDCs) generated from BM cultivated for 6 days in GM-CSF; lane 4: lysates of brain tissue. Quantitative numbers reflect an arbitrary assignment of BM4 = 1 following normalization of all DP58 levels to those of the housekeeping gene, G3PDH. C. Western blot analyses of DP58 expression in identical protein loads of cellular lysates as used for qPCR. Lane 1, lysates of fresh bone marrow cells; lane 2, lysates of BM4 cells (bone marrow cells exposed for 4 hrs to anti-DC reagent); lane 3, lysates of immature DCs (IDCs) generated from BM cultivated for 6 days in GM-CSF; lane 4, lysates of brain tissue; M, protein standard. Protein loading control measured in terms of tubulin level applied in each lane above.

4.3. Comparison of DP58 nucleotide sequences from brain and BM 4 cells

The PCR products derived from brain and bone marrow, using the DP58-specific primers were sequenced and shown to be identical. In addition, the sequences were identical to the corresponding region of the RIKEN cDNA identified as DP58 (NCBI database: NP_780664)

4.4. Demonstration of DP58 expression in brain by immunohistochemistry

Using the DP58 peptide-specific antibody described previously, we performed immunohistochemistry on brain tissue sections. The results in Figure 2A show DP58 immunoreactivity in all mouse brain regions. The nuclei of nerve cells were immunolabeled in all cortical layers (Figure 2A), in the pyramidal layer and the dentate granular layer of the hippocampal formation (Figure 2B), in the basal ganglia (Figure 2C) and brainstem (Figure 2D). A higher magnification revealed that the nuclear labeling consisted of a diffuse labeling of the entire nucleus and an intense labeling of inclusion bodies, approximately 5 μm in diameter (Figure 3). In the neocortex, the intense labeling appeared primarily at the periphery of the nucleus (Figure 3A). In the brainstem, the labeled bodies were more punctate in appearance and could also be seen in the perikaryon (Figure 3B). The same pattern of immunoreactivity was also seen in the cerebellum, where the nuclei of Purkinje cells were clearly labeled (Figure 3C), although the nuclei of granule cells were not. These results confirm that DP58 is a cytosolic protein in BM4

cells and suggest that DP58 may be synthesized in the neuronal perikaryon and subsequently transported into the nucleus. Tissues treated with pre-bleed serum instead of the primary antibody raised against DP58 showed no labeling.

DP58 protein was localized by immunocytochemistry in stimulated BM cells (BM4) and in cultured nerve cells. In Figure 4, BM4 cells were imaged by transmitted light (Figure 4A) for clear identification of cells. The nuclei of these BM4 cells were stained with the dye DAPI which stains dsDNA, (Figure 4B). Immunohistofluorescence of DP58 was detectable only in the cytosol of BM4 Pro-DC cells (Figure 4C). This was shown in a previous study by Western blotting (16). By contrast, DP58 was localized to the nuclei of cultured nerve cells labeled with the rabbit anti-DP58 antibody as shown in Figure 4D (arrowhead); MAP2, used for identification of nerve cells, was localized only to the dendritic processes (Figure 4D, arrows).

In order to confirm DP58 expression in the nuclear and cytoplasmic fractions of nerve and BM4 cells, we isolated these fractions, and subjected their lysates to SDS-PAGE and Western blotting as shown in Figure 5(Lanes 1, 2, 5 and 6). The whole brain and BM4 lysates were used as the positive control (Figure 5, Lanes 3 and 7). The Figure 5 reveals that DP58 band was discernible only in the nerve cell nuclei and in cytoplasmic fractions of BM4 cells. Tubulin served as a loading control for each lane.
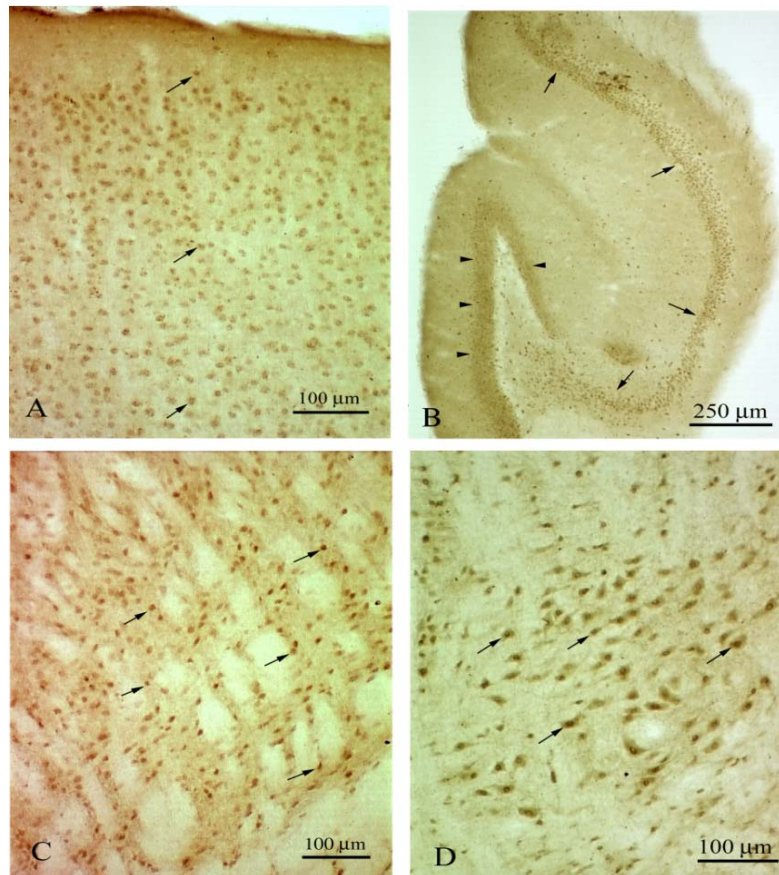
Figure 2. DP58 immunolabeling in the cortex (A), hippocampus (B), basal ganglia (C) and brainstem (D) of the mouse brain. In A, C and D, arrows point to representative immunoreactive neuronal nuclei. In B, arrows point to the immunoreactive pyramidal layer of the hippocampus and arrowheads to the granular layer of the dentate gyrus.
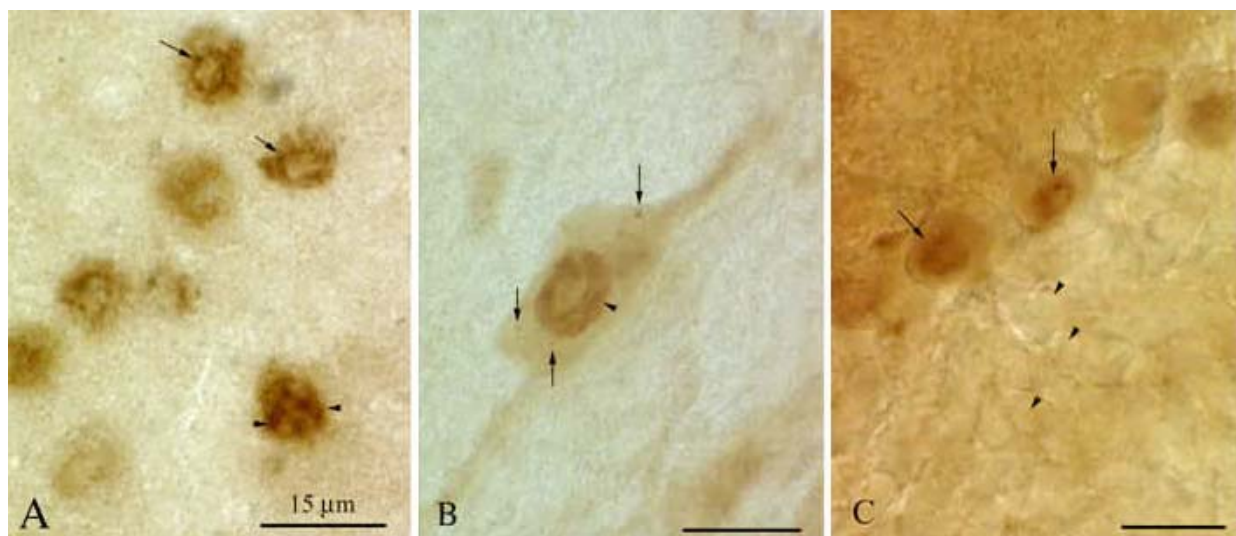

Figure 3. DP58 immunolabeling of neuronal nuclei in the cortex (A), brainstem (B) and cerebellum (C). Arrows in A point to the nuclear labeling and arrowheads to the particulate labeling. Arrows in B point to the particulate labeling in the neuronal perikaryon and the arrowhead to the nuclear labeling. Arrows in C point to the nuclear labeling in cerebellar Purkinje cells and the arrowheads to the non-immunoreactive granule cells. Bar equals 15 micrometers in all figures.
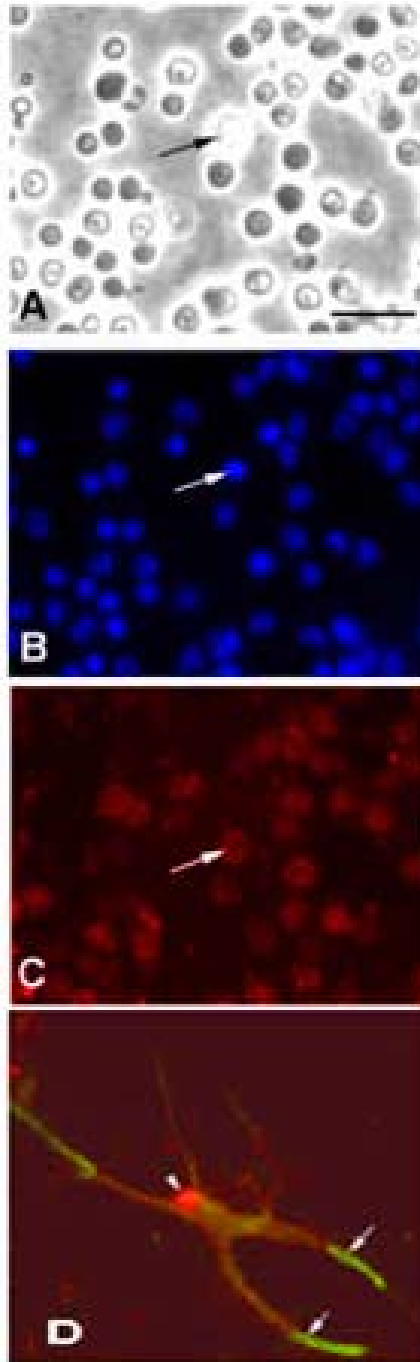
Figure 4. BM4 cells were processed for DP58 immunohistochemistry using a CY3 (red) fluorescence label. BM4 cells were imaged by transmitted light (A), by fluorescence for DAPI (B) and DP58-CY3 (C). The same BM4 cell is indicated by the arrow. Note that the nucleus of the indicated BM4 cell stains with DAPI (B) but not with DP58-CY3 immunolabel, which is cytosolic (C). (D), Confocal image of a cultured mouse neuron immunolabeled for DP58 and MAP2. Note that the DP58 immunolabel localized to the nucleus (red, arrowhead) and the MAP2 immunolabel to the neuronal processes (green, arrows). Bar equals 20 micrometers.

4.5. Post-translational modification of DP58 in different tissues

Since SDS-PAGE and Western blotting of DP58 from BM4 cells and brain revealed two distinct molecular species, 58 kDa and 52 kDa respectively (Figure 1C), it was of interest to determine if post-translational modification would account for the differences in molecular sizes. To demonstrate the phosphorylation status, we first prepared immunoprecipitates (IP) from lysates of brain tissues, naive BM, BM4 and DC cells using the affinity-purified anti-DP58 antibody reagent, and subjected them to SDS-PAGE. The analysis reveals only a single band in lanes corresponding to BM4 and brain preparations as shown by SDS-PAGE (Figure 6A). Further confirmation of the protein bands' identity was obtained by Western-blotting with rabbit anti-DP58 antibody as the primary antibody (Figure 6B).

In parallel experiments, the precipitated DP58 was Western blotted using commercial anti-phosphotyrosine, antiphosphoserine, and antiphosphothreonine antibodies. It is clear from Figure 6C, 6D and 6E that brain protein was phosphorylated mostly at serine residues. However DP58 in BM4 cells was phosphorylated at the tyrosine, threonine and serine residues. Whether the two isoforms have any other posttranslational difference is currently not known. Also specific amino acid residues involved in phosphorylation in DP58 and its isoform 52 kDa brain protein remain to be investigated.

5. DISCUSSION

Earlier data suggested that DP58, a novel inducible cytosolic protein, might be a specific phenotypic marker of early DC progenitors (16). The evidence presented in this study clearly shows that DP58 expression does not occur exclusively in BM-derived early progenitor DCs. Expression of this novel molecule is also observed in brain tissues. Using qPCR we have demonstrated that there is 1200 times more of DP58-specific mRNA in mouse brain than in BM-derived BM4 cells. However, BM4 cells express higher levels of DP58 protein than do brain tissues. Parallel sequencing of the products of PCR-amplified DP58 from brain tissue and BM4 cells reveals complete identity with the DP58 protein sequence (16).

Two interesting points emerge from analyses of the results of this study. First, of all tissues, only cells of the immune system and brain express DP58, tissues that are derived from different germ layers. Immunocytochemical and subcellular fractionation studies clearly indicate that DP58 is predominantly located in neuronal nuclei; whereas, in BM4 cells, it is cytosolic. The two proteins also differ in observed molecular weights. Furthermore, the 58kDa protein in Pro-DC, BM4 cells, is phosphorylated at tyrosine, threonine and serine residues; whereas, in neuronal cells it exists as a 52kDa protein also phosphorylated but mostly at serine residues. The apparent difference in size may be due to phosphate moieties in DP58. The other noteworthy point is that, although at the mRNA level, the brain tissues exhibit considerably higher

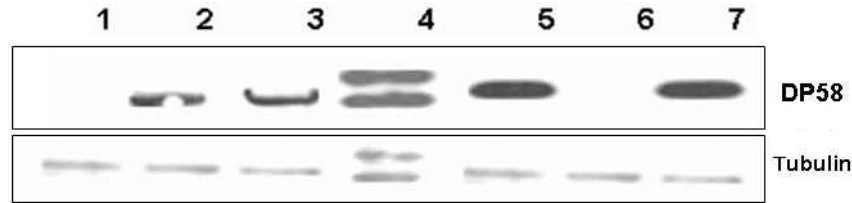**Constitutive expression of DP58 in brain nuclei**

Figure 5. DP58 protein expression in the nuclear and cytoplasmic fractions of Neurons and BM4 cells. Lane 1, brain cytosolic fraction; lane 2, brain nuclear fraction; lane 3, whole brain lysate; lane 4, protein standard; lane 5, BM4 cytosolic fraction; lane 6, BM4 nuclear fraction, and lane 7, whole BM4 cell lysate. Protein loading control measured in terms of tubulin level applied in each lane above.
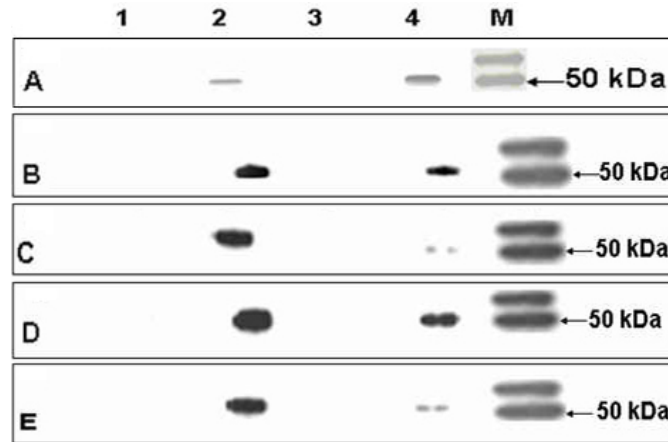

Figure 6. Demonstration of DP58 isoforms and their Phosphorylation status. The SDS-PAGE profile of Coomassie-Blue-stained bands from the immunoprecipitates (IP) of identical loads of of various lysates. (B) Western blot analyses of the same immunoprecipitates for DP58 protein expression using anti-DP58 as the primary antibody. (C-E) Western blot analyses of the same immunoprecipitates to assess DP58 phosphorylation status. The lanes are as follows: Lane 1, IPs from fresh bone marrow cells; lane 2, IPs from BM4 cells; lane 3, IPs from immature DCs (generated from BM cultivated for 6 days in GM-CSF); lane 4, IPs from brain tissues; lane 5, protein standard. C- 6E were generated using anti-phosphothreonine, anti-phosphoserine, and antiphosphotyrosine respectively as primary antibodies.

levels of DP58 than in BM4 cells, this does not happen proportionately at the protein level. Western blot analysis using the same protein amounts from brain and BM4 cells shows that the expression is highest in the latter. In BM-derived progenitors, the protein is induced during BM4 differentiation; whereas, in nerve cells from diverse regions such as cortex, brainstem, and basal ganglia, DP58 expression occurs constitutively. A low detectable level of cytosolic DP58 is, however, discernible in nerve cells by immunocytochemistry, but not by Western blotting, that was performed on enriched nuclear and cytosolic fractions of brain tissues and BM4 cells. This may be due to differences in sensitivity of the two techniques. Irrespective this difference, it is clear that both techniques confirm the differential expression of DP58 and its isoform in brain and Pro-DC, BM4 cells.

Quite unexpected was the observation that the detectable level of DP58 mRNA exhibits a significant discordance with the detectable level of DP58 protein both in bone marrow cells and in the adult brain. Using quantitative PCR we demonstrated that there is about 2.6 times as much DP58 mRNA in naïve bone marrow cells

compared to the same cells treated for 6 days with GM-CSF (BM4 cell Figure 1B). However, we are unable to detect any DP58 protein in naïve bone marrow cells, yet significant amounts of protein are found in progenitor BM4 cells (Figure 1C). It is possible that DP58 mRNA is translationally inhibited in naïve bone marrow and that the activation of BM cells during differentiation releases this mRNA for translation, thus accounting for the rapid rise in DP58 protein in progenitor BM4 cells. There are numerous examples of translational regulation of pre-existing mRNAs. During Xenopus and mouse oocyte maturation, maternal mRNAs become translationally active in a strictly controlled temporal order (25, 26). Alternatively, the DP58 mRNA could be sequestered from the translational machinery and upon stimulation with anti-DP58 antibody reagent the mRNA is transported to a translationally active subcellular location. Several mRNAs have been shown to be translationally controlled by regulated distribution (27).

Whether activation of naïve bone marrow leads to the release of DP58 mRNA from a translational inhibitory complex or its localization to the translational apparatus, the consequences must also render the mRNA

susceptible to RNase degradation accounting for the 2-3 fold decrease in the detectable amount of this mRNA following stimulation of naïve bone marrow cells. Within the adult brain a more dramatic discordance is observed. In this adult tissue, the DP58 mRNA level is 1200 times higher than in GM-CSF-induced bone marrow cells. However, the level of detectable brain DP58 protein in an equivalent amount of cellular protein is less than that in BM4 cells. This suggests that, within differentiated neural tissue, DP58 mRNA is highly stable yet translated at much reduced levels relative to bone marrow. We are currently investigating possible mechanisms that could account for these observations.

The differences in DP58 occurrence between brain and BM4 cells may be explained in terms of their differentiation status. BM4 cells represent differentiating DC progenitors, whereas neurons are adult quiescent cells unlikely to respond to any differentiation stimuli. Nuclear location of DP58 in neurons may reflect an anti-apoptotic property to maintain G_0 status in a manner similar to that observed with BCL3 proteins (23). However, why this is important only in brain but not in other tissues, and whether both cytosolic and nuclear forms of DP58 exist in a developing neuron need to be addressed. In other words, whether neurogenesis accompanies deployment of DP58 as an inducible phosphoprotein would be an important aspect of our future studies.

Although DP58 is a novel protein that has not been previously described, it does contain sequences corresponding to ankyrin repeats. Ankyrins were originally discovered as components of the erythrocyte membrane (23). They have a great variety of developmentally regulated, tissue-specific isoforms, which are involved in various cells and subcellular structures, such as in membrane skeleton organization and cell-cell adhesion regulation (24). It has been shown that the IkB family of proteins contains ankyrin repeat motifs apparently sufficient for interaction with NF kB proteins (22). In view of the fact that DP58 contains ankyrin repeat motifs and that it has the ability to locate in both cytoplasm and nucleus of specific cells depending on their differentiation status, it is tempting to suggest that DP58 may be another member of IkB family resembling proteins like BCL3, which can also exist both in the nuclei and cytoplasm. Whether this similarity based on ankyrin repeats can be used to regard DP58 as another nuclear-localizing signaling protein remains an interesting issue for future investigation.

6. ACKNOWLEDGEMENT

7. REFERENCES

1. Amico A.D. and Wu L. The early progenitors of mouse dendritic cells and plasmacytoid predendritic cells within the bone marrow hemopoietic precursor expressing flt-3. *J. Exp. Med* 198, 293-303 (2003)

2. Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y. J., Pulendran, B.and Palucka, K. Immunobiology of dendritic cells. *Annu. Rev. Immunol* 18, 767-811 (2000)

3. Ardavin, C. Origin, precursors and differentiation of mouse dendritic cells. *Nat. Rev. Immunobiol* 3, 582-591(2003)

4. Hess, D.C., Hill, W.D, Carroll, J.E. and Borlongan, C.V. Do Bone Marrow Cells Generate Neurons? *Arch. Neurol* 61, 483-485 (2004)

5. Bianco, P., Riminucci, M., Gronthos, S., Robey, P. G. Bone marrow stromal stem cells: nature, biology and potential application. *Stem cells* 19, 180-192 (2001)

6. Golub, E.S. Brain-associated stem cells antigen: an antigen shared by brain and hemopoietic stem cells. *J. Exp. Med* 136, 369-374 (1972)

7. Akeson, R., and Graham, K. L. A new antigen common to the rat nervous and immune system: 1 detection with hybridoma. *J. Neurosci. Res* 6, 165-177 (1981)

8. Jiang, Y., Naessen, B., Lenvik, T., Blackstad, M., Reyes, M., Verfaillie, C. M. Multipotent progenitor cells can be isolated from postnatal bone marrow, muscle, and brain. *Experimental. Hematol* 30, 896-904 (2002)

9. Morris, R. (1985) Thy-1 in developing nervous tissue *Dev. Neurosci Neurosci* 7, 133-160 (1985)

10 Avraham, S., London, R., Fu, Y., Ota, S., Hiregowdara, D., Li, J., Jiang, S., Pasztor, I. M., White, R. A., Groopman, J. E., and Avraham, H. Identification and Characterization of a novel related adhesion focal tyrosine kinase (RAFTK) from megakaryocytes and brain. *J. Biol. Chem* 46, 27742-27751 (1995)

11. Tan, J., Town, T., Mori, T., Obregon, D., Wu, Y., DelleDonne, A., Rojiani, A., Crawford, F., Flavell, R. A. and Mullan, M. CD40 is expressed and functional on neuronal cells. *The. EMBO. J* 21, 643-652 (2002)

12. Hao, H.N., Zhao, J., Thomas, R.L., Parker, R. L. and Lyman, W. D. Fetal human hematopoietic stem cells can differentiate sequentially into neural stem cells and then astrocytes in vitro. *J. Hematother. Stem Cell. Res* 12, 23-32 (2003)

13. Dustin, M. L.and Colman, D. R. Neural and immunological synaptic relations. *Science* 298, 785-789 (2002)

14. Czura, C.J. and Tracey, K. J. Autonomic neural regulation of immunity. *J. Intern. Med 257*, 156-166 (2005)

15. Goolsby, J., Marty, M. C., Heletz, D., Chiappelli, J., Tashko, G., Yarnell, D., Fishman P.S., Dhib-Jalbut, S., Bever, C. T., Pessac, B., and Trisler, D. Hematopoietic progenitors express neural genes. *Proc. Natl. Acad. Sci USA* 100, 14926-14931(2003)

16. Al-Shaibi, N., and Ghosh, S. K. A novel phosphoprotein is induced during bone marrow commitment to dendritic
 cells. *BBRC* 321, 26-30 (2004)

17. Graham, J. M. In Subcellular Fractionation - a practical approach (ed Graham, J. M. and Rickwood, D.), *Oxford University Press. Oxford. UK* pp 1-29 (1997)

18. Wiley, J., and Sons, Inc. Isolation and analysis of the proteins. Current Protocols in Immunology. *Chapter* 8.1-5 (2002)

19. King, M.W., Ndiema, M. and Neff, A. Anterior structural defect by misexpression of Xgbx-2 in early *Xenopus* embryos are associated with altered expression of cell adhesion molecules. *Dev. Dyn* 212, 563-579 (1998)

20. Brewer, G. J. Isolation and culture of adult rat hippocampal neurons. *J. Neurosci. Meth* 71, 143-155 (1997)

21. Mohn, KL, Laz T.M., Hsu, JC, Melby, A.E, Bravo, R., Taub, R. The immediate-early growth response in regenerating liver and insulin-stimulated H-35 cells: comparison with serum-stimulated 3T3 cells and identification of 41 novel immediate-early genes. *Mol. Cell. Biol*. 11, 381-390 (1991)

22. Bundy, D.L., and Mckeithan, T.W. Divers effects of BLC3 phosphorylation on its modulation of NF-KappaB p52 homodimer binding to DNA. *J. Biol. Chem* 272, 33132-33139 (1997)

23. Avis, J. Q, and Bennett, V. Brain ankyrin. A membrane-associated protein with binding sites for spectrin, tubulin and the cytoplasmic domain of the erythrocyte anion Chanel. *J Biol. Chem* 259, 13550-13559 (1984)

24. Hryniewicz-Jankowska, A., Czogalla, A., Bok E., and Sikorsk, Ankyrins, multifunctional protein involved in many cellular pathways. A. F. Folia. *Histochem. Cytobiol* 40, 239-49 (2002)

25. Charlesworth, A, Welk, J and MacNicol, A. The temporal control of Wee1 mRNA translation during Xenopus oocyte maturation is regulated by cytoplasmic polyadenylation elements within the 3' untranslated region. *Dev. Biol* 227, 706-719 (2000)

26. Tay, J, Hodgman, R and Richter, JD. The control of cyclin B1 mRNA translation during mouse oocyte maturation. *Dev. Biol* 221, 1-9 (2000)

27. St Johnston, D. Moving messages: the intracellular localization of mRNAs. *Nature. Rev. Mol. Cell. Biol* 6, 363-375 (2005)