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Evidence That Glycoprotein 96 (B2), a Stress Protein, Functions as a Th2-Specific Costimulatory Molecule

Pinaki P. Banerjee,2,* Dass S. Vinay,2,3† Ajith Mathew,* Manoj Raje,* Vrajesh Parekh,*
Durbaka V. R. Prasad,* Anil Kumar,* Debasis Mitra,* and Gyan C. Mishra4*7

After the engagement of Ag receptor, most of the Th cells for their optimal activation require a second (costimulatory) signal provided by the APCs. We demonstrate the isolation and characterization of a 99- to 105-kDa protein (B2), from LPS-activated B cell surface, and its function as a Th2-specific costimulatory molecule. Appearance of B2 as a single entity on two-dimensional gel electrophoresis and as a distinct peak in reverse-phase HPLC ascertains the fact that B2 is homogeneous in preparation. Electron microscopy as well as competitive binding studies reveal that 125I-labeled B2 specifically binds anti-CD3-activated T cell surface and also competes with its unlabeled form. Internal amino acid sequences of B2 are found to be identical with stress protein gp96. The identity of B2 as gp96 is also revealed by immunological characterization and by confocal microscopic colocalization studies of B2 and gp96 on LPS-activated B cells. Confocal imaging studies also demonstrate that gp96 can be induced on B cell surface without association of MHC molecules. Furthermore, the novel role of gp96 in Th cell proliferation skewing its differentiation toward Th2 phenotype has also been established. Ab-mediated blocking of gp96-induced signaling not only abrogates in vitro proliferation of CD4⁺ T cells, but also diminishes the secretion of Th2-specific cytokines. Notably, the expression of CD91 (receptor of gp96/B2) is up-regulated on anti-CD3-activated Th cells and also found to be present on Th1 and Th2 subsets. The Journal of Immunology, 2002, 169: 3507–3518.

The optimum activation of Th cells is dependent on the signals through the molecules expressed on the surface of the APCs. A number of APC-derived cell surface determinants have been shown to possess the ability to potentiate T cell effector functions. One of these signals, accounting for the specificity of the immune response, involves the engagement of the TCR by an appropriate peptide-MHC complex displayed by the APCs. However, Quill and Schwartz (1) had demonstrated that the occupancy of TCR alone is not sufficient to drive T cells to an optimum state of activation. Additional cell surface-derived costimulatory signals are also required in determining the fate of the ongoing immune response. In addition, a T cell that has received signal through the TCR must commit either to activation or anergy depending on the presence or absence of the costimulatory signals delivered by the APCs (2).

Depending on the source of activation, the CD4⁺ T cells were shown to secrete Th1-type cytokines (IFN-γ and IL-2), which can take part in cell-mediated immunity, or they can strengthen the humoral arm of the immune system by secreting Th2-type cytokines such as IL-5, IL-4, IL-6, and IL-13 (3). Thus, the nature of the costimulatory molecules and their regulation appears to be of considerable interest. Available data suggest that various receptor ligand pairs, expressed on the surface of T cells and APCs, have shown to possess costimulatory properties. These include ICAM-1/LFA-1 (4), VCAM-1/VLA-4 (5), HSA (6), B7-CD28 (7), B7.2-CD28 (8), and LFA-3/CD2 (9). Although it is considered that B7-CD28 interaction is central in the steps of T cell activation, studies on CD28-deficient mice (10) have shown that this may not be a limiting factor in T cell response. Furthermore, compelling evidence is being rapidly added to the existing literature about the involvement of hitherto unknown molecules with costimulatory functions, which also have the ability to initiate and propel the T cell response irrespective of the B7-CD28 pathway. To name a few, the 4-1BB (11), OX 40 (12), B7 RP (13), and B7-DC (14) are the latest addition to this list.

Working on strategies to find out additional molecules with T cell costimulatory properties, which not only activate the T cells but also show a distinct bias in Th response, we have earlier reported the identification and characterization of few novel costimulatory molecules present on the surface of APCs. Interestingly, a 150-kDa protein (M150) isolated from the membrane fraction of activated macrophages, the APC that is known to stimulate Th1 response, was shown to function as a Th1-specific costimulatory molecule (15, 16). Furthermore, two new costimulatory molecules, viz B1 (17) and B3 (18), were also identified on the surface of LPS-activated B cells and were found to elicit Th2 response. In continuation of our ongoing studies, we demonstrate in this study that the protein of 99–105 kDa, termed as B2, is expressed on LPS-activated B cells and specifically induces Th2 response from cultured CD4⁺ T cells. This protein (B2) was also found to be expressed on thioglycolate-elicited macrophages and bone marrow-differentiated dendritic cells (data not shown).
sequence homology between B2 and gp96, detailed biochemical characterization of B2, and codistribution of B2 and gp96 on LPS-activated B cell surface have revealed the identity of B2 as gp96. The significant role of gp96, in immunotherapy of cancers, has been well documented as it activates tumor-specific CTL responses (19). However, subsequent studies have also revealed that the gp96-engineered tumor rejection is not due to gp96 per se, but also emphasizes a direct link toward Th2 phenotype. Furthermore, the MHC-independent expression of gp96 on the APC has also been revealed by our confocal microscopic studies. Taken together, our study not only highlights a novel feature of the stress-related protein, but also emphasizes a direct link between stress and the regulation of immune responses.

Materials and Methods

**Mice**

Female BALB/c mice were obtained from the National Institute of Nutrition (Hyderabad, India). Mice were maintained in the experimental animal facility of National Center for Cell Science, India, and used at 6–8 wk of age.

**Medium**

CD4+ T cells, B cells, and A20 (B cell lymphoma) cells were cultured in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with penicillin (70 μg/ml), streptomycin (100 μg/ml), glutamine (4 mM), 2-ME (50 μM), sodium pyruvate (1 mM), HEPES (20 μM), and 10% heat-inactivated FCS (Life Technologies).

**Preparation of primary cells**

**B cells.** A single cell suspension of splenocytes were prepared in balanced salt solution (pH 7.2). The RBCs were depleted by hemolytic Gey buffer along with protease inhibitor mixture (10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 mM iodoacetamide, 10 μg/ml antipain, 10 μg/ml pepstatin, 10 μg/ml chymostatin, and 1 mM PMSF) and then subjected to a brief centrifugation at 700 × g for 10 min at 4°C to remove the nuclear fraction. The supernatant was centrifuged at 110,000 × g for 2 h at 4°C. The pellet was solubilized in buffer containing 1% Triton X-100, 20% glycerol, and 20 mM Tris-HCl (pH 7.5) for 8 h at 4°C, followed by centrifugation at 100,000 × g for 1 h at 4°C. The supernatant, containing membrane proteins, was separated using 10% SDS-PAGE. Isolation of the protein band of the interest was done as described in our earlier studies (17, 18). Briefly, protein band was located by staining a strip of the gel with silver nitrate, and the appropriate unstained portion was crushed and eluted with 1% SDS, 100 mM NH4HCO3, 50 mM Tris-HCl, 0.1 mM EDTA, and 150 mM NaCl (pH 8.0) at 37°C for 48 h. After filtration and centrifugation to remove the polycrylamide particles, the solution was dialyzed against 0.1% SDS, 10 mM NH4HCO3, 10 mM Tris-HCl, 0.1 mM EDTA, and 50 mM NaCl (pH 8.0) at 4°C for 24 h. SDS was removed using Extract-D-gel column (Pierce, Rockford, IL), and the protein was estimated using bicinchoninic acid kit (Pierce).

**Two-dimensional electrophoresis and reverse-phase HPLC**

The reagents for two-dimensional gel electrophoresis were prepared essentially according to Amory et al. (23) and performed as advocated by Penin et al. (24). For reverse-phase HPLC, isolated B2 molecule was diluted essentially with 0.5% trifluoroacetic acid and loaded onto a microbrobe HPLC column (C8 (Aquapore RP 300, Brownlee Columns; Applied Biosystems, Foster City, CA) and monitored.

**Protein sequencing**

Isolated B2 molecule was blotted onto ProteBlot (Applied Biosystems) in 3-[cyclohexamino]-1-propanol sulfonic acid buffer (pH 11.0), followed by staining with 0.2% Ponceau S and 1% acetic acid, and washed with PBS. The protein was then digested with trypsin, and a sample equivalent of 2 pnp from one of the digested fractions was subjected to internal sequencing up to 13 and 15 residues on Applied Biosystems (model 492 A) Procise Sequencer (at Protein Sequencing Facility, Worcester Foundation for Experimental Biology, Shrewsbury, MA).

**Negative staining of liposomes**

Liposomes were prepared by a reverse-phase evaporation method, as described elsewhere (17), and a sample droplet was picked up by touching a carbon-coated grid from 20 μl of aqueous liposome suspension. After allowing the excess liquid to drain off, the grid was gently dipped in 1% phosphotungstic acid (pH 7.0) and dried by blotting on a filter paper. Grids were observed in a transmission electron microscope (JEOL 1200 EXII, Tokyo, Japan), and representative fields were photographed.

**Coupling of iodinated B2 to liposomes**

125I labeling of B2 was performed using IODO bead method with PD-10 column (Pharmacia Biotech, Uppsala, Sweden). Iodinated samples were then precipitated by TCA. A 1:50 ratio of protein samples to liposomes was placed in a dialysis bag and dialyzed for 96 h at 4°C against 10 mM Tris (pH 8.0), 0.01 mM EDTA, and 10 mM NaCl with at least 12 changes. The contents of the dialysis bag were spun down for 2 h at 4°C at 178,000 × g. The pellet was then dissolved in PBS and later passed through a Sephadex G-50 mini column to remove unliposomized protein. Obtained sample (2 ml) was layered on top of a discontinuous gradient of 5–40% sucrose (w/v) in 10 mM Tris-HCl (pH 6.8), 150 mM NaCl, and 0.1 mM EDTA. The sample was then centrifuged overnight at 98,000 × g in a Beckman Coulter (Fullerton, CA) SW28 rotor at 4°C. A 2-ml sample was collected from the 5 and 10% interface, washed in 5 vol PBS by centrifuging at 178,000 × g for 2 h at 4°C. The pellet was dissolved in PBS and later used. The protein content coupled to liposomes was estimated, after lysis with 1% SDS, by the bicinchoninic acid method.

**Binding assay using electron-microscopic autoradiography**

The radiolabeled (25) and liposomized samples were incubated with CD4+ T cells (preactivated for 30 min with plate-bound anti-CD3 at 10 μg/ml concentration) or A20 cells for 30 min at 37°C. After incubation, the cells were washed thrice with cold PBS. To the pellet, an equal volume of low melting agarose (Sigma-Aldrich) was added and allowed to gel, and the latter was cut into 1-mm3 pieces. Trapped cells were fixed in 1% paraformaldehyde, 1% glutaraldehyde in PBS at 4°C for 1 h, followed by washing with PBS and postfixing in 1% osmium tetroxide for 90 min at 4°C in the dark. PBS-washed samples were passed through graded acetone series, and sections were embedded in epoxy resin (Bio-Rad, Hercules, CA). Sections cut on Ultratcut S (Vienna, Austria) were coated in darkness with photographic emulsion (Ilford Imaging (Paramus, NJ) nuclear L4 emulsion) and incubated in darkness for 2–3 wk in a dessicator at 4°C. The autoradiographs...
(25) were developed and stained with aqueous uranyl acetate and lead citrate, and were observed in a transmission electron microscope (JEOL 1200 EXII).

**Competitive binding assay**

For competition binding, a range of nonradioactive (cold) but liposomized dilutions of B2 was allowed to bind to the anti-CD3-activated CD4+ T cells. After washing, 125I-labeled B2 was added to a final volume of 200 μl and incubated for 2 h at 4°C with gentle agitation at 15-min interval. After extensive washing, the cell pellets were subjected to gamma counting (Beckman Coulter).

**Raising and purification of anti-B2 polyclonal Ab**

B2-specific peptide (GVVDSDDLPLNVsRE) as denoted by protein sequence was linked with keyhole limpet hemocyanin (KLH; Sigma-Aldrich) in the presence of 0.5% glutaraldehyde (1 mol peptide/50 aa of carrier proteins) in 2 ml reaction volume. After linking, the reaction was stopped with 1 M glycine. The peptide-KLH mixture then dialyzed for 12 h against PBS with repeated changes. A total of 150 μg of KLH-linked peptide was mixed with equal volume of CFA (Bangalore Genei, Pune, India) and immunized s.c. in 12-mo-old female rabbits. After 4 wk, the rabbits were boosted with 75 μg of Ag. Five days after first boost, anti-B2 antiserum was collected and anti-KLH Abs were removed by using anti-KLH Ab removal column (Bangalore Genei). The sera were further affinity purified using IgG purification kit (Bangalore Genei).

**Immunoblotting**

Cell lysate (15 μg) or immunoprecipitated samples were separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-Rad). The immunodetection was performed with an anti-B2 (affinity-purified polyclonal anti-B2 IgG) or anti-gp96 Ab (goat anti-gp96 C-terminal specific polyclonal Ab; Santa Cruz Biotechnology, Santa Cruz, CA) and with control Abs (normal rabbit IgG and normal goat IgG; Bangalore Genei), followed by HRP-coupled secondary Ab incubation (goat-anti-HRP, Santa Cruz Biotechnology, and anti-rabbit HRP, Bangalore Genei), and visualized using chemiluminescence substrate (Pharmacia Biotech). For our initial studies, anti-gp96 Ab was kindly provided by M. Green from St. Louis University School of Medicine (St. Louis, MO).

**Immunoprecipitation**

Ice-cold PBS-washed cells were lysed in lysis buffer containing 0.5% Triton X-100, 10 mM Tris-HCl (pH 8.0), and 140 mM sodium chloride at 4°C for 1 h. The cell lysate was centrifuged at 10,000 × g for 30 min at 4°C to remove the nuclear debris. To 100 μl of supernatant, related Ab was added either at the final concentration of 15 μg/ml or at mentioned dilutions. The Ab-supernatant mixture was incubated for 2 h at 4°C, followed by adding 20 μl of protein G-agarose (Life Technologies), and further incubated for 2 h at 4°C on a rocker. At 4°C, the slurry was washed twice with TSA buffer, followed by washing for 30 min at 4°C with required fluorescent-conjugated secondary Ab, cells were washed twice and fixed with 1% paraformaldehyde and then visualized with a confocal laser-scanning microscope (Zeiss, Jena, Germany) at a magnification of ×63.

**Surface labeling and confocal microscopy**

B cells (1 × 10^5) were harvested from cultures, washed, and suspended in FACS buffer (PBS with 2% FCS and 0.1% sodium azide). Cells were first treated with normal hamster sera to prevent any nonspecific binding of the Abs. Then, in FACS buffer cells were incubated with anti-gp96 (Santa Cruz Biotechnology) and/or anti-I-Ad PE Ab (BD Pharmingen) for 45 min at 4°C. After two washes with FACS buffer, followed by staining for 30 min at 4°C with required fluorescence-conjugated secondary Ab, cells were washed twice and fixed with 1% paraformaldehyde and then visualized with a confocal laser-scanning microscope (Zeiss, Jena, Germany) at a magnification of ×63.

For competitive binding between B2 and gp96, plate-bound anti-CD3-activated (10 μg/ml, 30 min) Th cells (1 × 10^5) were first fixed with 4% buffered paraformaldehyde for 20 min at room temperature, washed, and then incubated along with FITC-conjugated B2 (B2–FITC) and unlabelled gp96 (Immatics Biotechnologies, Tubingen, Germany). In parallel experiments, activated Th cells were also incubated with FITC-conjugated B2 along with anti-CD91 Ab (Progen, Heidelberg, Germany). Both the competitors were used at 35-fold molar excess concentration. OVA and B2 were conjugated to FITC following standard laboratory protocol.

Additional studies were performed to ascertain whether B2 is codistribut-
ated with gp96. For this, 1 × 10^5 LPS-activated B cells were incubated simultaneously with rabbit anti-B2 and biotinylated goat anti-gp96 poly-
clonal Ab (10 μg/ml) for 45 min at 4°C. Cells were washed and stained with a mixture of anti-rabbit FITC and streptavidin-PE for 30 min at 4°C. After incubation, cells were washed and visualized with a confocal laser-scanning microscope at a magnification of ×63.

Inhibitory binding studies between anti-B2 and anti-gp96 Ab were also performed on LPS-activated B cells. For this, cells (1 × 10^5) were first incubated with biotinylated goat anti-gp96 Ab (10 μg/ml) for 45 min at 4°C. After washing, the cells were then incubated with the rabbit anti-B2 Ab (10 μg/ml) and stained with anti-rabbit FITC. In parallel experiments, cells were first incubated with anti-B2 rabbit Ab (10 μg/ml) for 45 min at 4°C. After washing, the cells were then incubated with the biotinylated goat anti-gp96 Ab (10 μg/ml) and stained with streptavidin-PE.

T cell proliferation assay and quantitation of cytokines

Purified CD4+ T cells (1 × 10^5 cells/well) were cultured in the presence or absence of anti-CD3 (1 μg/ml; BD PharMingen) in 96-well round-bottom plate (Corning) in 200 μl final volume. The wells were precoated with anti-CD3 only when liposomized proteins were used in the culture. Otherwise, while using soluble anti-CD3, before adding the cells, wells were precoated with activated gp96 (20 μg/ml) and incubated at room temperature for 1 h. After washing, the cells were then incubated with the biotinylated goat anti-gp96 Ab (10 μg/ml) and stained with streptavidin-PE.

**Results**

A novel Th2-specific costimulatory molecule on activated B cells

Our approach for identifying the possible existence of the Th2-specific costimulatory molecules has been to resolve proteins from the membrane of activated APCs by gel electrophoresis, followed by elution of individual protein bands. To provide native context, each protein was reconstituted into lipid vesicles, and these reconstituted (liposomized) proteins were then tested for their costimulatory activity. B2 was identified, as a 99- to 105-kDa protein band, when LPS-activated membrane proteins of B cells were subjected to analyze by 10% SDS-PAGE (Fig. 1A). The homogeneity of the B2 was checked by two-dimensional gel electrophoresis, in which it appeared as a single spot (Fig. 1B). In addition, the same protein, when subjected to reverse-phase HPLC, showed a single prominent peak at 32.9 min in the chromatogram (Fig. 1C), demonstrating thereby that this protein preparation was homogeneous in its present form. Purified B2 was then reconstituted in liposomes, following which it was added in varying amounts to CD4+ T cells being cultured on anti-CD3-coated plates. As shown in Fig. 2A, liposomized B2 was indeed capable of inducing a dose-dependent proliferative response from T cells. Optimum proliferation, which was 20-fold more than the basal level, was observed with B2 concentration of 1 μg/ml, while higher doses of B2 showed an inhibitory effect (Fig. 2A). The specificity of this response is particularly evident from the fact that no such effect was observed in the several control groups that were set up in parallel and are shown in Fig. 2B. Also, this activity was not contingent upon its presentation...
in liposomized form, as similar results were obtained when the protein was used in immobilized form in T cell assays (data not shown). Controls such as anti-CD3 alone, liposomized B2 alone, or control liposome (not containing B2) did not influence the CD4+ T cell proliferation and lymphokine secretion over the basal level. However, as shown in Fig. 2C, mean of three independent experiments, costimulation with B2 resulted in a specific (near 10-fold) increase in the production of IL-4 and IL-5. As opposed to this, levels of IL-2 and IFN-γ in the same culture supernatants were virtually unaffected (Fig. 2C). These experiments, therefore, suggest that B2 is a costimulatory molecule that specifically induces the naive CD4+ T cells toward a Th2 phenotype.

**B2 binds specifically to the surface of activated T cells**

Our above observations of the costimulatory activity of B2 also suggested that the receptor for it must exist on the surface of T cells. To test this, we labeled B2 with 125I before its reconstitution in liposomes (Fig. 3, A and B), and the preparation was incubated with T cells, and binding, if any, was monitored by electron-microscopic autoradiography. As shown in Fig. 3C, the radiolabeled B2 incorporated in liposomes was indeed found to bind anti-CD3-activated T cells, being distributed uniformly along the cell surface, as denoted by arrows. As opposed to this, in the control experiment, no specific labeling of the T cells could be detected when liposome-incorporated 125I-labeled anti-mouse IgM was used for the binding studies (Fig. 3D). However, this later preparation was found to bind to A20 cells, as denoted by arrows (Fig. 3E). This result confirmed that the absence of such binding to T cells (Fig. 3D) was not due to any defect at the level of the liposome preparation, but the specificity of the interactions itself.

Specificity of the interactions between B2 and T cells was further confirmed in competitive inhibition experiments in which inclusion of excess unlabeled B2 diminished the binding of radiolabeled B2 in a dose-dependent manner (Fig. 4). These cumulative results, therefore, suggest the existence of receptor of B2 on the surface of the T cells. Another interesting feature of the data shown in Fig. 4 is the fact that the binding of B2 is optimal only in anti-CD3-activated T cells. In contrast, B2 binding to resting T cells was reduced by nearly 20-fold (Fig. 4). These findings therefore seem to imply that the putative receptor for B2 is up-regulated upon cross-linking of the TCR/CD3 complex.

**Identification of B2 as gp96**

To further characterize B2, we performed a proteolytic digestion with trypsin (as its N terminal was found to be blocked), and two of the fragments were subjected to the amino acid sequence analysis, as shown in Fig. 5. Interestingly, both of the obtained sequences exhibited complete identity with murine glucose-regulated protein of 94 kDa, or more commonly referred as gp96. The alignment of amino acid sequences of gp96 and B2 is shown in Fig. 5. This unanticipated relationship between B2 and gp96 has prompted further analysis. Therefore, to confirm the relationship between B2 and gp96, we next performed criss-cross immunoprecipitation experiments using lysates derived from a B cell lymphoma (A20). For these experiments, either a commercially available anti-gp96 Ab preparation or those generated against B2 peptide were used. When the A20 cell lysates were immunoprecipitated with either anti-gp96 or anti-B2 Ab, a protein band migrating at an identical molecular mass was obtained in both cases (Fig. 6A, lanes 1 and 7), whereas this band was absent when control IgG was used instead (Fig. 6A, lanes 3 and 8). The identical molecular mass of B2 or gp96 could further be evaluated by the position of standard protein molecular mass marker of 97.4 kDa (Fig. 6A, lane 6). Furthermore, when the supernatant of protein G-agarose-bound Ag-Ab (anti-gp96 polyclonal) complex was analyzed on the SDS-PAGE, any protein band equivalent to B2 mass could not be visualized even by silver staining (Fig. 6A, lane 2). As opposed to this, control Ab failed to immunoprecipitate the protein equivalent to B2 mass during the experiment (Fig. 6A, lane 4). The position of B2, as one of the SDS gel-separated proteins of A20 cell lysate, is shown in lane 9 of Fig. 6A. Importantly, anti-B2 Ab failed to immunoprecipitate any protein band from A20 lysates that had been first incubated or precleared with saturating concentration of anti-gp96 (Fig. 6A, lane 5). Conversely, preclearing the cell lysate with saturating concentration of anti-B2 was also found to deplete the gp96 protein, as determined in a subsequent immunoprecipitation analysis with anti-gp96 (Fig. 6A, lane 10). The striking homology between gp96 and B2 was further confirmed by immunoprecipitation, followed by Western blot experiments. Thus, the immunoprecipitated product obtained with anti-B2 was cross-reactive with anti-gp96 (Fig. 6B). Similarly, the protein product immunoprecipitated with anti-gp96 was also recognized by anti-B2 Ab (Fig. 6C).

In summary, the results in Figs. 5 and 6 strongly support that the protein B2, isolated from the membrane of LPS-activated B cells, was in fact the stress protein gp96.
Cell surface localization of gp96 and B2

As B2 was isolated from the surface of LPS-activated B cells and the essential prerequisite of a costimulatory molecule is that it should be expressed on surface of APCs, we therefore examined the expression of gp96 on activated B cells. LPS-regulated, nearly 10 times higher, cell surface expression of gp96 had previously been observed on murine B cells (26); however, it was essential to check its expression in the context of MHC molecule, as it had also been reported that gp96 being a tumor-specific transplantation Ag, carrying immunogenic peptide of the tumor cells, comes to the cell surface through MHC I (27). In addition, it had also been described that B cells were unable to present the gp96-Ag complex through MHC I (19). These observations caused us to examine the expression of gp96 on B cells in the context of MHC II. Our confocal microscopic studies revealed that gp96 could be expressed on the surface of the splenic B cells and is dependent on LPS stimulation (Fig. 7, upper and lower panel). We next investigated whether B2

**FIGURE 2.** B2 provides T cell costimulation and cytokine production. CD4+ T cells (1 × 10^5) were cultured in anti-CD3-coated wells for 60 h with various concentrations of liposomized B2 or with other controls. T cell proliferation was measured by [3H]thymidine incorporation during the last 12 h of culture. The data represent the mean ± SD of triplicates in one representative of at least three individual experiments. A, Dose-dependent effect of B2 on the proliferation of CD4+ T cells. B, The behavior of various controls such as anti-CD3 (10 μg/ml), PMA (10 μg/ml), B2 (1 μg/ml), liposomes (2.326 nmol inorganic phosphorous content), SDS (0.02 nmol), gel elute (10 μg/well), and LPS (1 μg/ml) on the proliferation of CD4+ T cells. C, B2 primes T cells to secrete Th2-type cytokines. Cultures were set up as described in the first paragraph of legend to Fig. 2. The cell-free supernatants were assayed as described in Materials and Methods. The values were expressed in terms of units per milliliter, derived from the standard curve.

**FIGURE 3.** Liposomized 125I-labeled B2 binds to anti-CD3-activated CD4+ T cell surface. A, Negative staining of liposomes by phosphotungstic acid (magnification ×10,000). B, Packing of radiolabeled B2 into liposomes vesicle as denoted by arrows (magnification ×20,000). C, Binding of 125I-labeled B2 on anti-CD3-activated CD4+ T cell surface (magnification ×40,000), as indicated by arrows. D, Negative control shows no binding of radiolabeled anti-IgM on CD4+ T cell surface (magnification ×20,000). E, Positive control, i.e., surface binding of 125I anti-IgM on A-20 cells, as denoted by arrows (magnification ×40,000). PM, plasma membrane; Cyt, cytoplasm; Nuc, nucleus (open bar = 200 nm).

**FIGURE 4.** Unlabeled B2 competes with its labeled form to bind on T cell surface. Either anti-CD3-preactivated or resting CD4+ T cells were incubated with different concentrations of liposomized 125I-labeled B2 in the presence or absence of liposomized unradiolabeled B2. The radioactivity by CD4+ T cells was monitored on a gamma counter. The data represent the mean ± SD of triplicates in one representative of at least three individual experiments.
FIGURE 5. Identical amino acid sequence of B2 and gp96. Two stretches of amino acid sequences obtained from B2 were subjected to homology search from the Atlas of Protein and Genomic Sequences (National Biomedical Research Foundation, Washington, D.C.). Number on the top of gp96 sequence denotes the position of that particular amino acid.

and gp96 are codistributed on LPS-activated B cells. Images obtained through confocal microscope showed the codistribution of B2 with gp96 (Fig. 8, A–D). Furthermore, the cell surface binding of anti-B2 Ab (Fig. 8E) was efficiently blocked (almost 80%) by anti-gp96 Ab, when the cells were preincubated with the anti-gp96 Ab (Fig. 8F). Similarly, preincubation with anti-B2 Ab considerably prevented the binding of anti-gp96 Ab to the LPS-activated B cells (Fig. 8, G and H). Experiments with control Abs did not show any effect on such competitive binding. Moreover, preincubation of cells with higher concentration of anti-B2 Ab completely abrogated the binding of anti-gp96 and vice versa. Thus, our results not only show the identical expression pattern of B2 and gp96 on LPS-activated B cells, but also depict colocalization of both the molecules, which unequivocally proves the identity between B2 and gp96. Furthermore, the expression of gp96 on the LPS-activated B cells was independent of the expression of MHC molecules, as most of the gp96 molecules were not colocalized with MHC II (Fig. 9). Thus, the results of confocal imaging, i.e., activation-dependent and MHC-independent surface expression of gp96 on B cell and its colocalization with B2, render indirect support to the possible role of gp96 as a costimulatory molecule.

FIGURE 6. Identification of B2 as gp96. A, Immunoprecipitated proteins from A20 cell lysate by both anti-gp96 and anti-B2 Ab as obtained by silver staining. Lane 1, A20 cell lysate was immunoprecipitated with anti-gp96 Ab. Position of gp96 on the 10% SDS gel is denoted by arrow. Lane 2, Supernatant of lane 1. Lane 3, Cell lysate immunoprecipitated with control Ab (normal goat IgG). Lane 4, Supernatant of lane 3. Lane 5, Cell lysate precleared with anti-gp96 Ab first and then immunoprecipitated with anti-B2 Ab. Lane 6, Protein molecular mass marker (97.4) in kDa. Lane 7, Cell lysate immunoprecipitated with anti-B2 Ab. Lane 8, Cell lysate immunoprecipitated with normal rabbit IgG. Lane 9, Position of B2 on the gel. Lane 10, Cell lysate precleared with anti-B2 Ab first and then immunoprecipitated with anti-gp96 Ab. B, Detection of B2 by anti-gp96 Ab. On a 10% SDS gel, A20 cell lysate, as positive control, was separated (lane 1), or immunoprecipitated samples at various concentrations by anti-B2 (lane 2–4) and with control Ab (lane 5) were run. The immunoprecipitated proteins were then transferred onto a nitrocellulose membrane, followed by immunoblotting with anti-gp96 in 1/1000 dilution. C, Recognition of gp96 by anti-B2 Ab. On a 10% SDS gel, immunoprecipitated samples by control Ab (lane 1) or by anti-gp96 Ab (lanes 2 and 3) were run along with the cell lysate of A20 cells (lane 4). The immunoprecipitated proteins and the separated cell lysate were then transferred on a nitrocellulose membrane, and immunoblotting was done with either anti-B2 (lanes 1 and 2) or anti-gp96 Ab (lanes 3 and 4, positive control) in 1/1000 dilution.

Th2-specific costimulatory property of gp96

As B2 and gp96 exhibited identical behavior and the B2 effectively costimulated the CD4+ T cells, we therefore were interested to know the possible role of gp96 in CD4+ T cell activation. Thus, to further elucidate the role of gp96 in T cell activation, commercially available plate-bound gp96 was tested for its ability to stimulate CD4+ T cells when anti-CD3 was used as the source of first signal. Interestingly, we found that gp96 is a potent costimulatory molecule and can activate the naive CD4+ T cells in a dose-dependent manner. An optimum level of activation, nearly 6-fold increment over the basal level was noted when gp96 was used at 10 μg/ml concentration. Furthermore, heat-inactivated gp96 failed to costimulate the Th cells, suggesting thereby that the costimulatory activity of gp96 is sensitive to thermal denaturation (Fig. 10A). Furthermore, blocking of the optimum costimulatory activity through gp96 by affinity-purified anti-B2 polyclonal Ab, at a concentration of 28 μg/ml, diminished the CD4+ T cell proliferation to the basal level. As opposed to this, control Ab (normal rabbit IgG), when used at the same concentration, was unable to abrogate such potency of gp96 (Fig. 10B). Thus, our results not only confirm the functional identity between B2 and gp96, but also assign a new costimulatory role to gp96.

After confirming the costimulatory activity of gp96, we then analyzed the cytokine profile obtained through such signaling. We assayed the signature cytokines for Th1 (IFN-γ) and Th2 type (IL-4, IL-10, and IL-13) as well. Interestingly, it was found that gp96 signaling was skewing the differentiation of naive Th cells only toward Th2 type; as IFN-γ, the hallmark of Th1 differentiation was not detected at all in the culture supernatants. Moreover,
diminishing the costimulation through gp96 either by using anti-B2 polyclonal Ab or by heat inactivating the protein also abolished the cytokine levels (Fig. 10C). Therefore, our experiments unequivocally prove that gp96 functions as a Th2-specific costimulatory molecule.

**Expression of CD91 on Th cells and its binding to B2/gp96**

The receptor of gp96 has been identified as CD91 (28), and results described in preceding sections show that B2 and gp96 are the same molecule. Also, electron-microscopic studies reveal that B2 binds to activated Th cells (Fig. 3). Therefore, we next examined whether such binding of gp96/B2 on T cells was mediated through CD91. Our results show that in comparison with a small population of resting Th cells (5–8%), FITC-tagged B2 binds to a 95–98% population of the anti-CD3-activated CD4+ T cells. Anti-CD91 Ab or unlabeled purified gp96 protein, at a 35 times higher molar concentration, abrogated this binding by 80% (Fig. 11), suggesting thereby that: 1) activated Th cells exhibit CD91, and 2) B2 and gp96 compete out each other for the same receptor (CD91).

We also tested as to whether Th1 and Th2 subsets express the CD91. For this, polarized Th cells were generated, and analysis of staining pattern through FACS demonstrated that both Th subsets express the receptor of B2/gp96 at comparable intensities (mean fluorescence intensities). However, in comparison with Th1 cells, a 10–15% higher population of Th2 cells binds to the fluorescent-tagged gp96 (Fig. 11, inset).
The data presented in this study describe, for the first time, the MHC-independent expression of gp96 on B cells. Images, at ×63 magnification, taken by confocal microscope show: phase-contrast image of a single LPS-activated B cell (A). B. Shows the same cell when incubated with goat C terminus-specific anti-gp96 polyclonal Ab, followed by staining with anti-goat FITC. The expression of MHC II molecules through the binding of anti-I-Ad PE on the surface of the same cell is shown in C. D. Shows overlapping images of B and C.

**Discussion**

The data presented in this study describe, for the first time, the costimulatory role of gp96 in the activation of CD4+ Th cells. It has also been observed that this stress protein activates Th cells to secrete cytokines solely representative of type 2 phenotype. The importance of stress proteins in immune regulation can be best addressed using Matzinger’s model of the immune response, i.e., danger theory of immunity (29). This model primarily focuses on the ability of the immune system to detect and to respond to the danger signals. This theory states that cell death from natural causes generate no stress signals. In contrast, if death occurs due to necrosis or ischemia, the cell undergoes a stress response with the liberation of stress protein-peptide complexes into the extracellular matrix (30) which functions as a danger signal to alert the immune system (31). Matzinger’s model of danger theory of immunity is based on three laws of T cell activation, which state: 1) resting T lymphocytes require both, i.e., Ag stimulation and costimulation with a danger signal for the activation process; 2) the costimulatory signal must be received through the APCs; and 3) the T cells receiving only Ag stimulation without the costimulatory signal undergo apoptosis.

Although it is difficult to catalogue the inventory of stress-mediated danger signals, however, a simplified view suggests that it could be just an invasion and physiological disturbances induced by pathogen, release of its metabolites, or endotoxin. Recently, the role of danger signal in the form of endotoxin (LPS) has been proven in the survival of memory T cells (32). Thus, to mimic the danger signal of the in vivo system, we cultured B cells with LPS and analyzed the capacity of such B cells to activate the Th cells. We found that Th cells cultured with anti-CD3 and LPS-activated B cells predominantly secrete the lymphokines representative of Th2 phenotype. Further analysis of such selective induction of Th cells by LPS-activated B cells led us to identify new Th2-specific costimulatory molecules (17, 18) from its membrane fraction.

In the present study, we describe the isolation and characterization of yet another molecule of 99–105 kDa, named B2, which provides Th2-specific costimulatory signal to the Th cells. It may also be emphasized in this work that the B2 alone at any given concentration does not stimulate the T cells, indicating that the biological effect of B2 is mediated only after cross-linking of the TCR. The homogeneity of the preparation of B2 protein not only was validated by reverse-phase HPLC and two-dimensional gel electrophoresis, but also had been categorically proven by the sequencing data, as the sequence analysis of two of the B2 peptides showed full identity with the sequence of a single protein, i.e., gp96. Compelling literature is available indicating the role of gp96 in immunotherapy of cancers. Retarded progression of primary cancers, reduced metastatic load, as well as a prolonged life span have been observed in tumor-bearing animals when treated with gp96 purified from autologous cancer cells (33). Furthermore, expansion of NK cell population and gp96-mediated elicitation of MHC I-restricted, tumor-specific CD8+ T lymphocytes has been noticed when the patients were immunized with the autologous cancer-derived gp96 preparation (34). In these studies, the role of gp96 has largely been attributed to its ability as carrier of immunogenic cancer cell-derived peptides. Of particular importance is the fact that the peptides associated with gp96 have been shown not to be MHC restricted, thereby being capable of cross-priming for CTL responses (35). It is also demonstrated that both macrophages and dendritic cells, when pulsed with the gp96 derived from tumor cells, have become potent activators of tumor-specific CTL responses, which may be again through an activation of CD4+ T cells (33). However, it may also be emphasized here that a tumor-derived gp96-Ig secretory fusion protein can directly activate specific CD8+ T cells, in the absence of any assistance from either macrophages or CD4+ T cells (36). Moreover, peptide-striped gp96 purified from tumor cells has also been shown to be capable of directly activating CD8+ T cells both in vivo and in vitro (21). Thus, these results collectively suggest that gp96 indeed plays an important role in T cell activation, the mechanism of which is yet to be established. So, as the internal sequences of B2 showed full identity with gp96, we have used classical immunological approaches to verify whether or not B2 and gp96 are identical. Our approach was based on the protocol described by Schaff et al. (37), in which the authors identified a 97-kDa protein as gp96 not only by its sequence homology with gp96, but also through immunodetection by anti-gp96 Ab. Interestingly, results of our immunodetection experiments with anti-gp96 Ab coupled with sequence analysis of B2 conclusively proved that B2 is indeed gp96.

Our experiments regarding the surface localization of gp96 (the probable costimulatory molecule) on APC also show that, only after LPS activation, gp96 tends to accumulate at the cell periphery and also on the surface of B cells. Glycoprotein 96 has been categorized as a member of heat shock protein (hsp) 90 and hsp83 family, respectively, in mice and *Drosophila*, and fluorescent microscopic studies with heat-stressed *Drosophila* cells also revealed parallel observation with respect to the localization of hsp83 (38). As far as negligible expression of gp96 on resting B cells is concerned, our data are strengthened by the fact that other authors have also reported negligible expression of gp96 on unstimulated splenic B cells of *Xenopus* and catfishes (39). Furthermore, negligible expression of gp96 on the surface of resting B cells is in confirmation with the fact that resting B cells are poor APCs and thus do not express the threshold level of most of the costimulatory molecules unless activated. In the foregoing account, confocal microscopic studies on the expression patterns of B2 and gp96 on LPS-activated B cells reveal the colocalization of these two molecules, which further supported the identical nature of gp96 and B2. Validating the results of confocal microscopy, results obtained by FACS analysis also showed near about same expression pattern of B2 and gp96 molecule on various cell lines viz A20, J77A, and P815. However, no detectable level of expression of either of these two molecules was found on EL-4 cell line (data not shown). One of the pilot studies regarding the surface localization of gp96 has revealed its expression only on transformed (Meth A induced fibrosarcoma) cells (40). However, it has also been postulated that due to the presence of C-terminal KDEL (lysine-aspartic acid-glutamic acid-leucine) sequence, which is the common retention signal for endoplasmic reticulum proteins (41), the majority of this
protein stays as a residual form in the endoplasmic reticulum (42). Only under stressful conditions, gp96 tends to redistribute to the Golgi apparatus (43), found to be enriched to some extent in the nucleus (44) and also to the outer surface of the plasma membrane (45). Costimulatory molecules are largely characterized by: 1) MHC-independent surface expression on APCs, and 2) ability to work crossing the MHC barrier. Significantly, our finding regarding the expression of gp96 on murine B cells in the context of MHC suggests that the expression of this stress protein is also independent of the expression of MHC molecules. Moreover, validating the second property of costimulatory molecule, interestingly enough, the surface expression of gp96 has also found to be phylogenetically conserved (39). Furthermore, the ancestral immunological role of gp96 as danger-signaling molecule (39, 46) has recently been outlined in the literature. Thus, time and again, the expression of gp96 on cell surface (40, 45), especially on B cells (26, 39), had been shown. However, no definite role to this molecule had been assigned yet. So, based on our previous results and reported data, we hypothesize that being a family member of one of the most primitive hsp, i.e., hsp90, gp96 may have originated as the first ever known danger-signaling molecule, as it is also found on some specific cells of the agnathan (hagfish), which lacks an adaptive immune system (39). Furthermore, this function of danger signaling (46), as a signal between innate and adaptive immunity, the molecule has been possibly retained through the evolution. Thus, to further elucidate the role of gp96 in the T cell activation, we have examined the role of this molecule as the source of accessory signal. Interestingly, gp96-mediated proliferation of CD4⁺ T cells was only seen when the cells were cultured in the similar condition of TCR occupancy and gp96,
which also lead the differentiation of Th cells toward Th2 phenotype by inducing the secretion of IL-4 and IL-13. Furthermore, it would be also worth mentioning in this work that the secretion of IFN-γ in undetectable level could be due to the inhibitory effect of IL-10 on IL-12 and IFN-γ production (47).

For an effective signal transduction, a costimulatory molecule should bind to its counter ligand on the target cell. Therefore, further studies were undertaken to explore this possibility, and the results obtained through electron-microscopic autoradiographic and competitive inhibition studies indicate the higher expression of a putative receptor of this molecule only on anti-CD3-activated T cells because no binding of $^{125}\text{I}$-labeled B2 beyond the basal level had been found when unstimulated naive T cells were incubated with it. Furthermore, parallel experiment by Singh-Jasuja et al. (48), in the search for binding of fluorescent-labeled gp96 on unstimulated splenic T cells, has also resulted in similar observations. Because the receptor of gp96, designated as CD91 (28), has recently been described on dendritic cells and macrophages (49, 50) and costimulation of Th cells requires receptor-ligand interaction, a question therefore arises as to whether Th cells express the same receptor for gp96 or not. Our experiments to answer this question resulted in an observation that, like the expression of inducible costimulator (51) and CTLA-4 (52), the expression of gp96 receptor (CD91) on Th cells was also found to be activation dependent. This observation, regarding the expression of CD91 on T cells, is further supported by the fact that the expression of CD91 has also been recently reported on a population of T cell thymoma, i.e., EL-4 cells (53). Interestingly, expression of CD91 was found to be present on Th1 as well as Th2 subsets with only a marginal increase (10–15%) in the Th2 population expressing CD91. Our observation regarding no preferential expression of CD91 on Th1 and Th2 subsets could possibly be explained by the fact that generation of a Th response and its maintenance and/or regulation are two immunologically distinct events. The former solely depends upon the strength of signal, i.e., the level of TCR interaction along with the level of CD28 signaling and the cytokine milieu (54), which denote the mode of activation and in turn the fate for the differentiation of naive Th cells. To exemplify, for activation of Th cells, an increasing concentration of plate-bound anti-CD3 had proven to have the synergic effect with the increasing concentration of anti-CD28 Ab, and simultaneous signaling through ICAM and B7.1 had also resulted in a more potent Th response (55). Moreover, it had also been found that the generation of Th2 subset from naive Th cells needs higher strength of signal in comparison with the strength required the generation of Th1 cells (54, 56), which does not hold true for the maintenance of either of these Th subsets. For example, although signaling through CD28 to naive Th cells and the generation of a Th2-biased response, by the production of Th2-specific cytokines such as IL-4 (57), had been documented in the literature (54, 58, 59), nevertheless, it had also been demonstrated that after polarization CD28-mediated signaling is not essential for the maintenance of Th2 cells (60, 61). Furthermore, engagement of the first signal (ionomycin) alone was proven to be sufficient to activate the Th2 cells, whereas for the activation of Th1 cells a combined signal by ionomycin and PMA was found to be essential (62). In continuation, it has also been demonstrated that IL-1, but not IL-4, plays a pivotal role for initiating the maintenance of Th2 subset and could either be secreted by the macrophages or even could be produced by the signaling through CD28 to the Th2 subsets (63). This IL-1-mediated signaling is also demonstrated to be independent of B7-CD28 interaction (63) and proven to be Th2 specific, as the Th1 cells do not express the IL-1R on them. In contrast, costimulation through CD28 to Th1 subsets generates IL-2 production, which in turn participates in their maintenance (63).

Thus, even though CD28 may be found to be present on both the polarized Th subsets (63), however, the requirement and/or outcome of signaling through it might be entirely different (60, 63, 64). In light of these observations, it is not surprising to find the ligand of gp96 on Th1 and Th2 cells. However, it is a matter of further investigation to reveal the extent of requirement of gp96-mediated signaling and its outcome on differentiated Th1 and Th2 cells, more importantly in a scenario of stress-mediated skewing of immunoresponse from Th1 to Th2 kind (65). Because stress stimuli such as heat shock, glucose starvation, or LPS activation (26) induce the up-regulation of gp96 and the costimulatory activity of gp96 also governs the Th response toward Th2, therefore, it would
be postulate to that the molecule like gp96 might regulate this shift of Th response. Moreover, during in vitro CD4+ T cell proliferation assay (data not shown), glucose-deprived or heat-shocked APCs also generated a more potent Th response than the unstimulated group. Thus, the present observations depicting the role of gp96 in T cell activation probably denote a sort of parallelism between stress and costimulation.

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