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A Novel Protein MAJN Binds to Jak3 and Inhibits Apoptosis Induced by IL-2 Deprival

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To find a possible signal interacting with the Jak3 N-terminal, we screened the human peripheral blood cDNA library through both a two-hybrid system and a tyrosine-phosphorylation-modified two-hybrid system using the N-terminal of Jak3 as bait. Results showed that one new homologue of myosin heavy chain, designated MAJN (molecule associated with Jak3 N-terminal), could bind to Jak3 in a tyrosine-phosphorylation-independent manner. The interaction between Jak3 and MAJN was further confirmed by immunoprecipitation in BAF-B03 β cells. To investigate the function of MAJN, we have constructed the BAF-B03 β/MAJN cell line that stably expresses MAJN and found that overexpression of MAJN can partially inhibit the apoptosis induced by interleukin-2 deprival. Further studies are needed to elucidate how MAJN executes its function to antagonize BAF-B03β cell death in the absence of IL-2. © 2000 Academic Press

Key Words: **two-hybrid system; tyrosine-phosphorylation-modified two-hybrid system; interleukin-2 (IL-2); Jak3 N-terminal (JN); molecule associated with Jak3 N-terminal (MAJN); apoptosis.**

Binding of cytokines to their receptors induces oligomerization of cytokine receptors and initiates a cascade of intracellular signaling events that result in the proliferation and differentiation of many cells, particularly those of the immune system (1). Although cytokine receptors such as interleukin 2 receptor (IL-2R) lack intrinsic kinase activity, they associate with and activate cytoplasmic protein tyrosine kinases Jaks that sequentially phosphorylate downstream signaling molecules such as the signal transducers and activators of transcription (STATs) (2).

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The Janus family of kinases (Jak1, Jak2, Jak3, Tyk2) is well known by the existence of tandem kinase and pseudokinase domains (3). Like the Roman god of gates and doorways, Jaks are "two-faced." In addition, five other segments of homology were recognized among Jaks, designated JH3–JH7. Jak3 constitutively binds to IL-2R γ through N-terminal including JH3–JH7 region and transmits IL-2 signal to downstream target genes, such as induction of c-*fos*, c-*myc*, *tnf-*β, ultimately leading to cytokine-mediated cell proliferation (4–7). Though our previous studies have demonstrated that Jak3 induced *tnf-*β transcription though Stat 5 activation (6), the mechanism of c-*myc* and c-*fos* induction by Jak3 activation is not well established. Therefore, it is necessary to find signalers involved in the different pathways leading to cell proliferation or anti-apoptosis.

It is reported that the interaction between Jak3 N-terminal and IL-2R γ cytoplasmic domain is critical for IL-2 induced cell survival and cell growth (8, 9). In addition to their interaction with cytokine receptor chains, recent data have demonstrated that Jak3 directly interacts with Jab through JH1 kinase domain and with Stat5 through JH2 pseudokinase domain (10, 11). In contrast, the function of JH3–JH7 is relatively limited to the interaction with IL-2Rγ.

To find other signals interacting with the JH3–JH7 region of Jak3 and to help further clarify the functions of Jak3, we utilized a two-hybrid system to screen a human peripheral blood cDNA library. Recently, evidences have accumulated to demonstrate that tyrosine-phosphorylation plays a crucial role in protein-protein interaction (12, 13). Therefore, in order to find molecules that interact with Jak3 dependent of tyrosine-phosphorylation, we utilized the modified two-hybrid system by co-expression of Src kinase to strengthen the necessary tyrosine-phosphorylation level. In identifying N terminal of Jak3-interacting proteins by using yeast two-hybrid system and Tyrosine-phosphorylated-modified system, we obtained one homologue of myosin heavy chain (MHC),

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designated MAJN (molecule associated with Jak3 N-terminal), which interacted with Jak3 independent of tyrosine-phosphorylation level. Functional analysis has demonstrated that MAJN can partially inhibit the apoptosis induced by IL-2 withdrawal in BAF-B03 β cells.

MATERIALS AND METHODS

Yeast two-hybrid system. A PCR fragment encoding the C-terminal of Jak3 (JH3–JH7) was cloned into the vector pUC6S by using *Hin*dIII and *Bgl*II, and then cloned into pBTM116 (kindly provided by Di Zhang, Albert Einstein Medical College) and pBTM116 Src (generously provided by Jonathan A. Cooper) downstream from the Lex A binding domain, termed pBTM116 JN and pBTM116 Src/JN. Deletion mutants of Jak3 N terminal used for two-hybrid system assay were as follows: JH2-4 represents region of Jak3 from JH2 to JH4, JH5-7 is JH5~JH7, JH4-7 is JH4~JH7, JH3-5 is JH3~JH5, and JH3-6 is JH3~JH6 (see Fig. 2). After co-transformation of yeast host strain L40 cells with pBTM116 Src/JN and human peripheral blood cDNA library (Clontech), the selection was performed on Trp-, Leu-, and His-deleted plates with 5 mmol/liter 3-AT to repress the His leakiness. Physical interaction between Jak3 and MAJN was measured by filter color assay (14). Briefly, yeast transformants were transferred onto nitrocellulose membranes, permeabilized in liquid nitrogen, and placed on Whatman 3MM papers that had been soaked in Z buffer containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside(X-gal). After developing at 37°C for 10 h, the yeast cells forming either blue or white colonies were classified into positive (+) or negative group (−), respectively. Photographs were also utilized to illustrate the interaction.

Cell culture and plasmid construction. BAF-B03 cell line is kindly provided by Dr. Yufang Shi from Holland, which is a subline of the IL-3-dependent murine hematopoietic cell line BA/F3. It endogenously expresses IL-2R α and γ_c chains but not IL-2R β chain (15). BAF-B03β is a derived cell line stably expressing IL-2R β chain and survives in the presence of IL-2. The plasmid pCDNA3-c-Myc-MAJN was constructed by cloning the c-terminal of MAJN into pCDNA3-c-myc. The open reading frame is confirmed to be right though DNA sequencing. After transfection with pCDNA3-c-Myc-MAJN, BAF-B03β cells were incubated for 36 h and then added 400 μg/ml G418 to select the monoclone cell line BAF-B03 β/MAJN appeared.

Immunoprecipitation and immunoblot analysis. BAF-B03 β cells (1×10^7) transfected with the plasmid pCDNA3-c-Myc-MAJN were incubated for 48 h. Cells were then collected and solubilized in 1× loading buffer and cell lysate was used for Western blot analysis. In immunoprecipitation experiment, cells were solubilized with RIPA buffer (0.5% Nonidet P-40, 0.1% sodium desoxycholate, 0.1% SDS, 10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride). Immunoprecipitates obtained with anti-*c*-MYC (Santa Cruz Biotechnology) were resolved by SDS/PAGE, and then transferred to PVDF membrane. Filters were probed with anti-JAK3 (Santa Cruz Biotechnology). Blots were visualized with the ECL detection system (Amersham).

Quantitation of cell death. The percentage of dead cells was quantitated by the trypan blue dye-exclusion method (16). After starved of IL-2 for indicated times, BAF-B03 β/MAJN monoclone No. 5 and No. 6 and control cells BAF-B03 β were harvested, and then resuspended in medium lacking IL-2. Subsequently, 100 μl of a 0.5% trypan blue solution was mixed with 100 μl of the cell suspension. Viable and nonviable cells were counted on a hemocytometer with a minimum of 500 total cells counted.

DNA fragmentation assay. Apoptotic DNA fragments were prepared according to a modification of previously described (17). Briefly, 1×10^6 cells were harvested and washed with PBS for two times, and then lysed with 0.2 ml lysis buffer containing 10 mM Tris, 150 mM NaCl, 10 mM EDTA, 0.4% SDS, 1 mg/ml proteinase K (pH 8.0). After overnight incubation in a 55°C water bath, the lysate was extracted with phenol and phenol/chloroform (1:1), respectively. The DNA was then precipitated with ethanol. One-third of the DNA sample was applied onto a 1.5% agarose gel and visualized by staining with 500 ng/ml ethidium bromide.

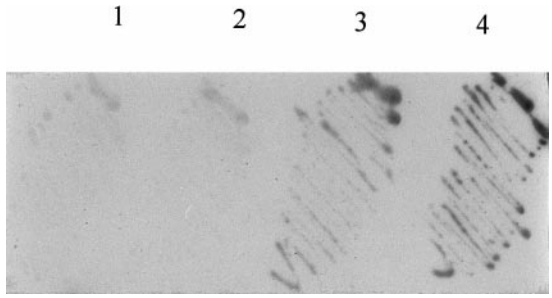

FIG. 1. MAJN specially binds to Jak3 N-terminal in a tyrosine-phosphorylation-independent manner in a two-hybrid system. Different plasmids were transfected into yeast host strain L40 and the activities of β-galactosidase in various transformants were measured to illustrate the interaction between MAJN and Jak3 N-terminal. 1, pBTM116 and pGAD10-*MAJN;* 2, pBTM116-*Src* and pGAD10-*MAJN;* 3, pBTM116-*JN* and pGAD10-*MAJN;* 4, pBTM116-*Src/JN* and pGAD10-*MAJN*.

RESULTS AND DISCUSSION

Identification of MAJN as a protein interacting with the N-terminal of Jak3. After screening a yeast two-hybrid human peripheral blood cDNA library with pBTM116-JN and pBTM116-Src/JN, we obtained 10 positive clones from a total of 3×10^6 independent clones. After sequencing, we found that they encoded three different proteins (18, 19). One of them is the C-terminal of an unidentified protein, designated MAJN (molecule associated with Jak3 N-terminal), which is homologous to myosin heavy chain. Retransformation experiment has demonstrated that yeast harboring C-terminal of MAJN and N-terminal of Jak3 displayed high β-galactosidase activities no matter Src kinase is cotransfected or not (Fig. 1). These data demonstrated that MAJN could interact with Jak3 N-terminal in a Tyrosine-phosphorylation-independent way in two-hybrid system.

The fragment that we obtained encoded the C-terminal of MAJN (GenBank Accession No. D86970), from 1389 to 1581 aa. Except for myosin tail motif composed of coiled-coil structure, there is no typical function motifs found in the c-terminal of MAJN that we identified to associate with Jak3. Therefore, we assume that MAJN possibly bind to Jak3 N-terminal through its coiled-coil region.

Delineation of the N-terminal of Jak3 interacting with MAJN using a two-hybrid system. To describe the region of Jak3 binding to MAJN in detail, we co-

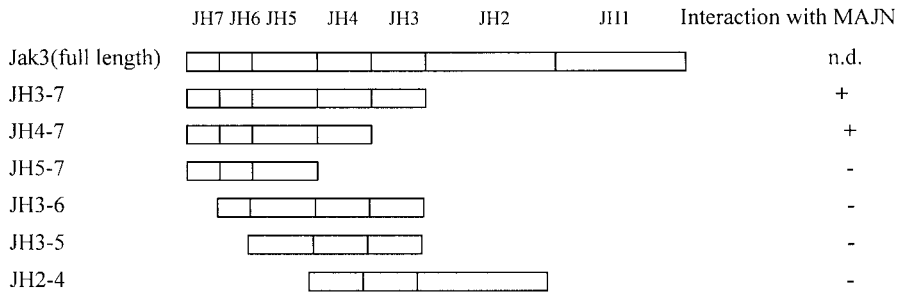

FIG. 2. Construction of Jak3 deletion mutants and detection of the interaction between various Jak3 mutations with MAJN in a two-hybrid system. The structures of various Jak3 mutants are shown. The interaction between Jak3 mutations with MAJN was measured by filter color assay (14).

transformed the various deletions of the Jak3 N-terminal and MAJN into yeast L40 and then detected their β-galactosidase activities, respectively.

As shown in Fig. 2, results showed that the region of Jak3 including JH4-JH7 has the ability to associate with MAJN, while other mutations including JH2-4, JH5-7, JH3-5, and JH3-6, which disrupted the integrity of JH4-JH7, all lost the ability to interact with MAJN.

Coimmunoprecipitation of Jak3 with MAJN in BAF-B03 β cells. To further confirm the interaction of Jak3 with MAJN, we cloned MAJN into pCDNA3-c-Myc as a C-terminal fusion with 6 copies of c-myc epitope, designated pCDNA3-c-Myc-MAJN. And then we transfected it into BAF-B03 β cells. Due to the endogenous expression of Jak3, we did not cotransfect the Jak3 expression vector into BAF-B03 β cells. After 48 h incubation in the presence of IL-2, cells were collected and lysis buffer was added to solubilize cells. The cell lysate was immunoprecipitated with anti-cMyc antibody and sequentially resolved by 6% SDS/PAGE. Western blot was performed with antibody toward Jak3. As shown in Fig. 3, anti-Jak3 specially recognized one band in cells expressing MAJN, while the control was not. Our results demonstrated that the interaction between MAJN and Jak3 indeed exists in the mammalian cells.

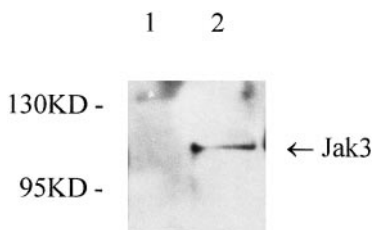

FIG. 3. MAJN specially interacts with Jak3 in BAF-B03 β cells. Control vector pCDNA3 cMyc (lane 1) and pCDNA3 cMyc-MAJN (lane 2) was transfected into BAF-B03 β cells respectively and the cell lysate was incubated with agarose conjugated c-myc antibody. The precipitates were resolved in SDS/PAGE and Western blotted with anti-Jak3 antibody.

Overexpression of MAJN partially inhibits the apoptosis induced by IL-2 deprival. Northern blot results demonstrated that MAJN was specifically expressed in thymus and peripheral blood leukocytes (data no shown), suggesting that MAJN possibly play an important role in hemopoietic cell activity. To study the function of MAJN, we constructed the cell line BAF-B03 β/MAJN that stably expresses MAJN using G418 selection.

After construction of stable cell line, we performed Western blot to verify the expression of MAJN using anti-cMyc antibody. Unexpectedly, we found that anti-cMyc specially recognized one band with a molecular mass of about 70 kDa, which indicated that C-terminal of MAJN was prone to homodimerize (Fig. 4A). The phenomenon can be explained by the structure of C-terminal of MAJN, which is myosin tail and composed of the coiled-coil structure. To confirm this suppose, we transfected MAJN into Cos7 cells transiently and performed Western blot. Western blot result from Cos7 cells is consistent with that of stable cell line BAF-B03 β/MAJN (Fig. 4A).

BAF-B03 β cells proliferate and sustain its viability dependent of IL-2 (20). It is reported that deprival of IL-2 can induce apoptosis (21). On the other hand, T cells from Jak3-deficient mice have an increased susceptibility to undergo apoptosis (22), which indicated a critical role of Jak3 in the process of apoptosis. To study the function of MAJN or its interaction with Jak3 in IL-2 signal transduction, we used trypan blue staining test assay to see if MAJN can compensate the function of IL-2. The results showed here are very interesting. The apoptosis percent of BAF-B03 β/MAJN is obviously less than that of control cells after IL-2 withdrawal, which implied that MAJN expression could sustain the cell viability (Fig. 4B). DNA fragmentation assay was also performed to confirm the anti-apoptosis function of MAJN. Results revealed that after 12 h starved of IL-2, chromosome DNA mixture extracted from BAF-B03 β/MAJN cells exhibited lower density of DNA ladders in contrast to control (Fig. 4C). These data illustrated that MAJN overexpression in-

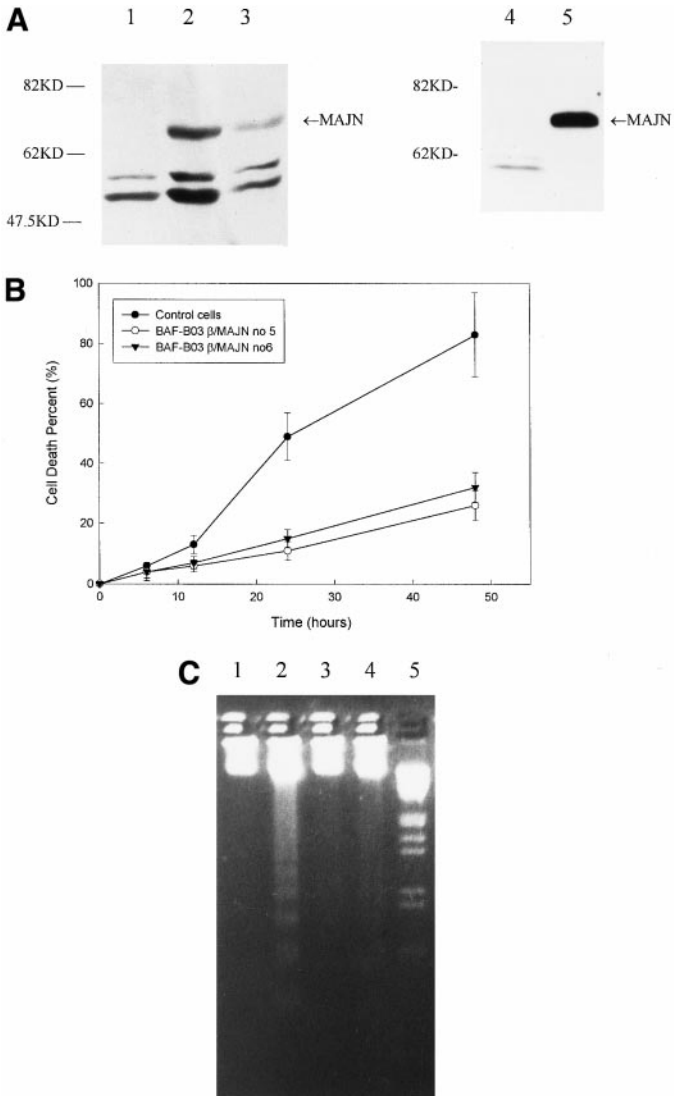

FIG. 4. Overexpression of MAJN in BAF-B03β can partially inhibit the apoptosis induced by IL-2 withdrawal. (A) Construct of stable cell line BAF-B03 β/MAJN which expresses MAJN. Cell lysate extracted from control cells (lane 1) and BAF-B03 β/MAJN monoclone no. 5 (lane 2) and no 6 (lane 3) cells were blotted with anti-cMyc antibody. Cos7 cells transiently transfected with pCDNA3-cMyc (lane 4) and pCDNA3-cMyc-MAJN (lane 5) were analysis using Western blot assay with anti-cMyc antibody; (B) effect of MAJN expression on cell death as quantitated by the trypan blue exclusion. BAF-B03 β/MAJN monoclone no 5 and no 6 and BAF-B03 β control cells were starved of IL-2 for 6, 12, 24, and 48 h, respectively. The percent of apoptotic cells was counted as described under Materials and Methods. (C) DNA fragmentation analysis of BAF-B03 β/MAJN (lane 4) after deprival of IL-2 for 12 h in comparison with control cells BAF-B03 β (lane 2). Lane 1 and lane 3, respectively, comes from control cells and BAF-B03 β/MAJN cells which is incubated in the presence of IL-2. DNA maker was also shown in lane 5.

deed has the ability to antagonize the apoptosis induced by IL-2 withdrawal.

Recently, it was reported that a CED-4-homologous protein FLASH is involved in Fas-mediated apoptosis, while its c-terminal can inhibit this apoptosis after auto-degradation (23). It gives us some guide in this way. Our results demonstrated that C-terminal of MAJN play a critical role in IL-2-mediated antiapoptosis function. However, we do not know if the full-length MAJN can inhibit the apoptosis or not. The successive research will proceed with the potential function of full-length MAJN.

In addition, IL-2 deprivation triggers apoptosis in the murine T cell line TS1alphabeta, a process that can be blocked by overexpression of Bcl-2 (24). All three Ras proteins, K-, N-, and H-Ras, interact with Bcl-2; however, their mitochondrial localization is differentially regulated in IL-2-supplemented or -deprived cells. These results indicated that different localization of Ras might play a crucial role in the apoptosis. In our research, MAJN possibly inhibit the apoptosis through the similar mechanism to alter some important molecules localization and sequential activation, such as bcl-2, Ras, Akt, and so on (24, 25). Therefore, our following research will also concentrate in the study about the relationship between MAJN with these apoptosis-related factors to clarify the molecular mechanism of the novel protein.

In conclusion, we have used a two-hybrid system to identify one novel molecule MAJN that associates with Jak3 N-terminal. MAJN overexpression can rescue BAF-BO3 β cells from apoptosis after the removal of IL-2. Further research will focus on the anti-apoptosis mechanism to answer this question: How does MAJN execute its function to antagonize the apoptosis induced by IL-2 deprival?

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