Vol. 200, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1994 Pages 443-449

IDENTIFICATION OF AN ALTERNATIVELY SPLICED FORM OF THE MURINE HOMOLOGUE OF B7

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Received February 6, 1994

SUMMARY: B7 on antigen presenting cells is a costimulatory ligand necessary for full activation of T cell. Receptors for B7 have been known as CD28 or CTLA4. We here show that in addition to B7 mRNA, an alternatively spliced mRNA (designated as MB7-2 mRNA), that immunoglobulin (Ig)C-like domain coded by exon3 has been spliced out, is found in activated murine splenic B cells by reverse transcriptase-polymerase chain reaction analysis. Chinese hamster ovary (CHO) cells transfected with MB7-2 bound CTLA4Ig less well than those expressing B7, but bound CD28Ig to a similar extent, indicating that IgV-like domain contains the complete binding site for CD28. In addition, IgC-like domain may participate in an increase in the affinity for CTLA4. Thus, MB7-2 represents a new form of the murine B7 with different receptor binding properties.

Although occupancy of the T cell receptor (TcR) complex by antigen in conjunction with the MHC is necessary for the initiation of T cell activation, several lines of evidence suggest that a costimulatory signal by antigen presenting cells is essential for the induction of optimal proliferation and lymphokine secretion (1,2). The occupancy of TcR in the absence of appropriate costimulatory signal results in nonresponsiveness, T cell anergy (3). Several recent studies provided compelling evidence that B7 and B70 (or B7-2) can provide such costimulatory signals (4-9). B7, a member of the immunoglobulin (Ig) superfamily, is a ligand for the T cell surface molecules, CD28 (10) and CTLA4 (11). The B7 molecule consists of a V-like domain (amino acids 38-143) and a C-like domain (amino acids 194-237) in its extracellular domain and has a 10 to 20-fold higher affinity for CTLA4 than for CD28 (11,12). CD28 and CTLA4 are also members of Ig superfamily

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0006-291X/94 $5.00

(10,11). Suboptimal activation of T cells with anti-CD3 or phorbol ester, together with crosslinking of CD28 by anti-CD28 antibodies or B7 ligand results in enhanced T cell proliferation and greatly augments synthesis and secretion of lymphokines (5,13,14). The blocking of B7/CD28 interaction and subsequent CD28 signalling by a soluble form of CTLA4 inhibit a variety of immunological responses (11,15-17) and prevents allo- or xenogeneic transplant rejection, and induces long term, antigen-specific unresponsiveness in vivo (17,18). Thus, it appears that an interaction between B7 and CD28 or CTLA4 provides a costimulatory signal important for various T cell responses. In this regard, the analysis of molecular and structural basis for the interaction between B7 and CD28 or CTLA4 is important.

In this study, we provide an evidence for the existence of an additional ligand for CD28 and CTLA4. CHO cells were transfected by an alternative spliced form of murine B7 (MB7-2) gene and wild type B7 gene. By using those transfectants, we analyzed the binding capacity and binding affinity of CD28Ig and CTLA4Ig to B7 and MB7-2. The results indicate that Ig V-like domain of B7 and MB7-2 provide the binding site for CD28, whereas IgC-like domain of B7 participate in an increase in the binding affinity for CTLA4 but not for CD28.

MATERIALS AND METHODS

Mice. C57BL/6 mice were obtained from Japan CLEA Inc., Tokyo, Japan and housed in a specific pathogen-free animal facility at Institute of Immunological Science, Hokkaido University.

Monoclonal antibody (mAb). Anti-murine B7(3A12) mAb was kindly supplied by Dr. Y. Liu, Department of Pathology and Kaplan Comprehensive Cancer Center, New York University Medical Center, NY (18).

Oligonucleotides for polymerase chain reaction (PCR). Oligonucleotides used in this study were synthesized based on published murine B7 cDNA sequence (12); sense primer, 5'CTAAGCTCCATTG-GCTCTAGATTC3' (nucleotide numbers-60 to -40); antisense primer, 5'CTCATGAGCCACATAATACCATGT3' (complementary to 944 to 967). Primers for beta actin (19) were also used to assess the integrity of RNA preparations.

Reverse transcriptase (RT)-PCR. Total cellular RNAs were prepared from lipopolysaccharide (LPS)-stimulated spleen cells by the method of Chomczynski and Sacchi (20). RT-PCR was performed as described by Saiki et al. (19). Briefly, 3 μg of total RNA were transcribed in a 60 μl reaction mixture containing 1X RT buffer (GIBCO BRL, Gaithersburg, MD), 60 units of RNase inhibitor (Promega Corp., Madison, WI), 300 units of Mo-MLV reverse transcriptase (GIBCO BRL), 1 mM of each dNTP (Pharmacia, Uppsala, Sweden) and 5 μM of random primer (Takara Shuzo Co., Ltd., Kyoto, Japan). An 0.4 μl aliquot of cDNA was mixed with 19.6 μl of PCR mixture containing 1X PCR buffer (Promega), 200 μM of each dNTP, 1 μM of each primer, 1 unit Taq DNA polymerase (Promega). PCR was performed using a programmed

temperature control system PC-700 (ASTEC, Fukuoka, Japan) set for 30 cycles under a condition as follows: denaturation at 94 ℃ for 1 min; annealing at 60 ℃ for 1 min; extension at 72 ℃ for 2 min. An 10 μl aliquot of each reaction mixture was analyzed by electrophoresis on a 1.5% agarose gel (Nippon Gene, Tokyo, Japan).

Isolation and sequencing of PCR products. PCR products were isolated from agarose gel by using a GENECLEAN kit (BIO 101, La Jolla, CA) and was directly subcloned into pCR II vector (Invitrogen, San Diego, CA). The nucleotide sequence of the subcloned DNA was determined by the chain termination method (21) using Sequenase ver. 2.0 DNA sequencing kit (USB, Cleveland, OH) and M13 reverse primer and M13 forward primer (Takara Shuzo Co. Ltd., Tokyo, Japan).

Cell culture and transfectants. B7 as well as MB7-2 cDNA were obtained from EcoRI digested pCR II vector described above, and subcloned into pcDLSRα296 expression vector (the plasmids were designated as B7-SRα and MB7-2-SRα, respectively). One million CHO cells were cotransfected with a mixture of 15 μg of expression plasmid clone B7-SRα or MB7-2-SRα and 1 μg of pAdD26SVA#3 carrying a dihydrofolate reductase (DHFR) gene using a Lipofectin reagent (Bethesda Research Laboratories, Gaithersburg, MD) following the methods supplied by vender (4). CHO cell clones expressing murine B7 or MB7-2 were selected by multiple rounds of fluorescence-activated cell sorting following immunostaining with a soluble form of CTLA-4 and are referred to as B7-CHO and MB7-2-CHO, respectively.

Recombinant proteins. A fusion protein consisting of the extracellular domain of human CTLA4 or CD28 and Ig Cγ1 domain was produced in CHO or COS cells, respectively and purified as previously described (4, 15). These proteins were referred to as CTLA4Ig and CD28Ig, respectively.

Immunostaining and FACS analysis. Transfected cells were analyzed by indirect immunostaining. Before staining, cells were removed from their culture flasks by incubation in PBS containing 0.5 mM EDTA. Cells were first incubated with mAb or Ig fusion protein in PBS for 1-2 h at 4° C followed by PE- or FITC-conjugated goat anti-human Ig Cγ1 serum (Tago Corp., Burlingame, CA) or F(ab)'2 fragment of FITC-conjugated goat anti-hamster Ig (CALTAG Laboratories Inc., South San Francisco, CA). Fluorescence on a total of 10,000 cells was then analyzed by FACScan (Becton Dickinson & Co., Mountain View, CA).

RESULTS

RT-PCR analysis of B7 and MB7-2 gene expression in LPS-stimulated spleen cells. RT-PCR was carried out to analyze B7 gene expression in LPS-stimulated spleen cells using a pair of primers shown in Materials and Methods. As reported in a previous publication (12), we consistently obtained wild type B7 cDNA (1030 base pairs;bp) in LPS-stimulated spleen cells from C57BL/6 mice. An additional PCR band (which is designated as MB7-2 hereafter) of approximately 280 bp shorter than B7 band was detected in LPS-stimulated spleen cells. MB7-2 gene expression was not detected in non-stimulated spleen cells. However, B7 gene expression was occasionally detected in non-stimulated spleen cells. Representative results are depicted in Figure 1A.

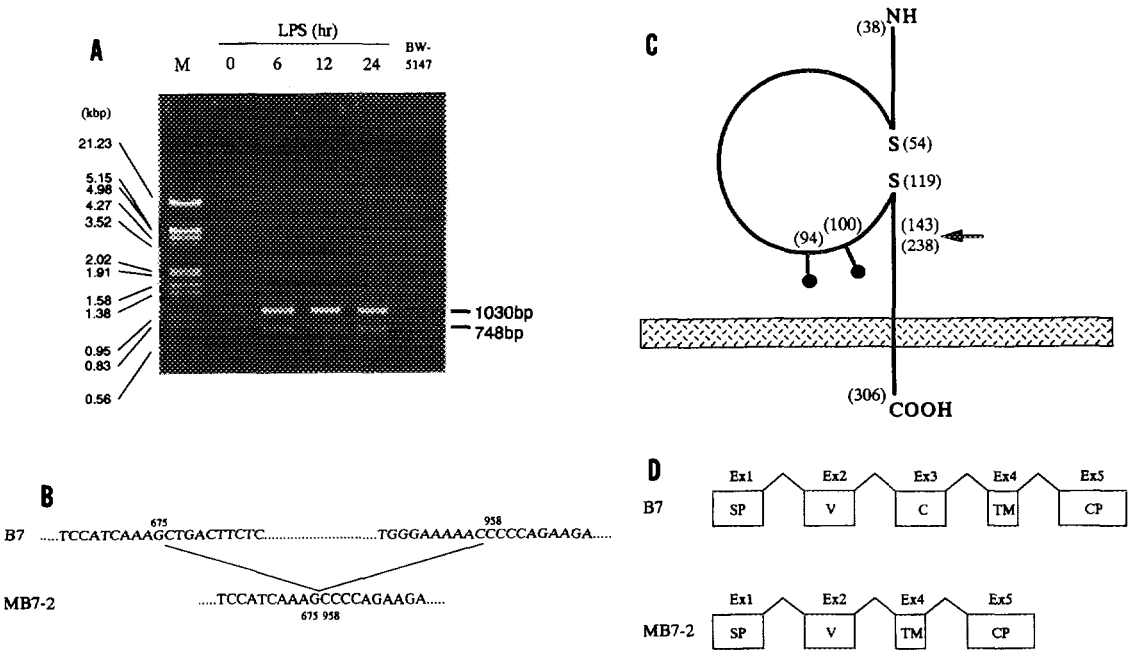

Fig. 1. (A) Detection of B7 as well as MB7-2 gene expression in LPS-stimulated B cells by RT-PCR. Spleen cells were cultured with 25 μg/ml LPS for indicated times. RNA was obtained from LPS-stimulated cells and BW5147 cells (as a negative control). (B) Nucleotide sequence of the MB7-2 and B7 PCR products at a spliced point. The upper line gives the sequence for B7, while the lower line gives that of MB7-2 PCR products. (C) Proposed structure of MB7-2. An arrow indicates the position where IgC-like domain is spliced out. Number indicates the corresponding position of amino acid of wild type B7. ♀ indicate the position of possible glycosylation. (D) Genomic organization of murine B7. SP; signal peptide. Ex; exon. V; V-like domain. C; C-like domain. TM; transmembrane domain. CP; cytoplasmic domain.

Cloning and sequencing of MB7-2. We isolated PCR products amplified from LPS-stimulated B cell cDNA of C57BL/6 mice, subcloned into pCR II vector and sequenced. The PCR band of normal size was found to correspond to the wild-type B7 cDNA. The sequencing of cDNA indeed exhibited complete identity to B7 cDNA (data not shown). The PCR band of smaller size (MB7-2) is an alternatively spliced form of B7 in which the C-like domain (from No. 144 amino acid to No. 237 amino acid) in its extracellular domain is spliced out (Fig. 1B). Thus amino acid No. 143 is fused to No. 238 amino acid in MB7-2 as shown in Fig. 1C. According to the genomic organization of murine B7 gene (22), the exon 3 of murine B7 is spliced out in MB7-2 (Fig. 1D). The signal peptide, transmembrane, and cytoplasmic domains of MB7-2 are identical to that of B7. Ig hinge like sequence is conserved in MB7-2. There are seven possible N-linked glycosylation sites at amino acids 94, 100, 150, 156, 190, 211, and 215 in the

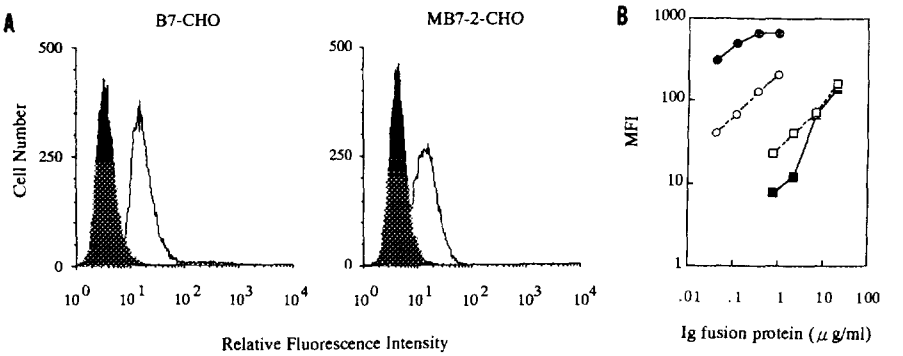

Fig. 2. (A) Binding of anti-B7 mAb to B7-CHO and MB7-2-CHO cells. Cells (1 x 10^6) were stained by saturating concentrations of anti-B7 mAb (3A12) followed by FITC-labeled second antibody. Samples were analyzed by flow cytometry. □And ■ indicate the positive staining and control staining with first mAb omitted, respectively. (B) Binding of CTLA4Ig and CD28Ig to B7- and MB7-2-CHO cells. Cells were (1x 10^6) first stained by various concentrations of CTLA4Ig or CD28Ig followed by PE-labeled second antibody. B7-CHO, CD28Ig (—■—); B7-CHO, CTLA4Ig (—●—); MB7-2-CHO, CD28Ig (-□-); MB7-2-CHO, CTLA4Ig (-○-). The data are representative of four experiments with similar results.

extracellular domain of B7, whereas there are only two possible N-linked glycosylation sites at amino acids 94 and 100 in the extracellular domain of MB7-2.

Binding activity of CTLA4Ig and CD28Ig to B7- and MB7-2- transfected cells. Two transfectants were used in this study. As shown in Fig. 2A, anti-murine B7 mAb (3A12) bound to B7-CHO and MB7-2-CHO transfectants to a similar extent. We also used anti-B7 mAb (1G10; Pharmingen, San Diego, CA) and found that 1G10 at saturating concentrations bound to B7-CHO < 1.5 fold greater than MB7-2-CHO in terms of mean fluorescence intensity (data not shown). These findings suggest that the number of B7 and MB7-2 molecules on the transfected CHO cells are comparable. The binding of purified CTLA4Ig and CD28Ig to B7-CHO and MB7-2-CHO cells were compared in the experiment shown in Fig. 2B. Transfected CHO cells were incubated with increasing concentrations of CD28Ig or CTLA4Ig, and binding was detected by flow cytometry. The number of CTLA4Ig molecules bound to B7-CHO was > 10 fold greater than that bound to MB7-2-CHO cells as judged by the intensity of fluorescence. The difference between B7-CHO and MB7-2-CHO in the binding of CTLA4Ig was maintained when aliquots of the cells were incubated with serial dilutions of the fusion protein. In contrast, the binding of CD28Ig to MB7-2-CHO and B7-CHO cells was comparable. It should be noted that CTLA4Ig has higher affinity than CD28Ig for MB7-2-CHO.

DISCUSSION

It was previously shown that interaction between B7 on antigen presenting cells and CD28 or CTLA4 on T cells provides a costimulatory signal for various T cell responses in vitro and in vivo (5,10-12,13-17). The extracellular portion of B7 consists of a Ig V-like domain and a Ig C-like domain, whereas that of CD28 and CTLA4 consists of a single Ig like domain. However, the molecular and structural basis for the interaction between B7 and CD28 or CTLA4 by Ig-like domains remains unclear.

By using a RT-PCR, we have analyzed B7 gene expression in mice. The important finding is that in addition to full length B7 mRNA, an additional PCR band (designated as MB7-2 mRNA) is found in activated murine B cells. MB7-2 is an alternatively spliced form of B7 in which the C-like domain is completely spliced out. Thus, MB7-2 possesses a single IgV-like domain in its extracellular portion and the transmembrane as well as cytoplasmic portions are identical to those of B7. The analysis of binding capacity of MB7-2 to CD28 and CTLA4 facilitates the understanding of role of IgV-like domain of B7 and MB7-2. The major finding is that the Ig V-like domain of MB7-2 and B7 contains the binding site for CD28, and the lack of IgC-like domain does not significantly influence the binding affinity for CD28. It is known that B7 has a 10 to 20-fold higher affinity for CTLA4 than for CD28 (11). The results of the present study demonstrate that MB7-2 also has a significantly higher affinity for CTLA4 than for CD28 (Fig. 2). It appears that IgV-like domain of B7 and MB7-2 has higher affinity for CTLA4 than for CD28. More importantly, IgC-like domain of B7 may participate in an increase in the affinity for CTLA4, but not for CD28 since CTLA4 had substantially higher affinity for B7 than for MB7-2.

Thus, MB7-2 represents a new form of the murine B7 molecule with different binding properties to their ligands.

ACKNOWLEDGMENTS. This work was partly supported by a Grant-in-Aids from the Ministry of Education, Science and Culture of Japan (Grant No. 04557020) to Toshimitsu Uede. We thank Laura Grosmaire, Bill Brady, Jennifer Johnson and Jeff Bradshaw for excellent technical assistance. We thank Dr. Y. Liu for providing anti-murine B7 (3A12) mAb. The sequence data of MB7-2 was deposited to EMBL/GenBank/DDBJ under accession number D16220.

REFERENCES

1. Hawrylowicz, C.M., and Unanue, E.R. (1988) J. Immunol. 141, 4083-4088.
2. Kawakami, K., Yamamoto, Y., Kakimoto, K., and Onoue, K. (1989) J. Immunol. 142, 1818-1825.
3. Schwartz, R.H. (1990) Science(Wash. DC). 248, 1349-1356.
4. Linsley, P.S., Brady, W., Grosmaire, L., Aruffo, A., Damle, N.K., and Ledbetter, J.A. (1991) J. Exp. Med. 173, 721-730.
5. Gimmi, C.D., Freeman, G.J., Gribben, J.G., Sugita, K., Freedman, A.S., Morimoto, C., and Nadler, L.M. (1991) Proc. Natl. Acad. Sci. USA. 88, 6575-6579.
6. Azuma, M., Ito, D., Yagita, H., Okumura, K., Phillips, J.H., Lanier, L.L., and Somoza, C. (1993) Nature (Lond.). 366, 76-79.
7. Hathcock, K.S., Laszlo, G., Dickler, H.B., Bradshaw, J., Linsley, P.S., and Hodes, R.J. (1993) Science (Wash. DC). 262, 905-907.
8. Freeman, G.J., Borriello,F., Hodes, R.J., Reiser, H., Hathcock, K.S., Laszlo, G., McKnight, A.J., Kim, J., Du, L., Lonbard, D.B., Gray, G.S., Naddler, L.M., and Sharpe, A.H. (1993) Science (Wash. DC). 262, 907-909.
9. Freeman, G.J., Gribben, J.G., Boussiotis, V.A., Ng, J.W., Restivo Jr., V.A., Lonbard, L.S., Gray, G.S., and Nadler, L.M. (1993) Science (Wash. DC). 262, 909-911.
10. Linsley, P.S., Clark, E.A., and Ledbetter, J.A. (1990) Proc. Natl. Acad. Sci. USA. 87, 5031-5035.
11. Linsley, P.S., Brady, W., Urnes, M., Grosmaire, L.S., Damle, N.K., and Ledbetter, J.A. (1991) J. Exp. Med. 174, 561-569.
12. Freeman, G.J., Gray, G.S., Gimmi, C.D., Lombard, D.B., Zhou, L.J., White, M., Fingeroth, J.D., Gribben, L.G., and Nadler, L.M. (1991) J. Exp. Med. 174, 625-631.
13. Reiser, H., Freeman, G.J., Razi, W.Z., Gimmi, C.D., Benacerraf, B., and Nadler, L.M. (1992) Proc. Natl. Acad. Sci. USA. 89, 271-275.
14. Razi, W.Z., Freeman, G.J., Galvin, F., Benacerraf, B., Nadler, L., and Reiser, H. (1992) Proc. Natl. Acad. Sci. USA. 89, 4210-4214.
15. Linsley, P.S., Wallace, P.M., Johnson, J., Gibson, M.G., Greene, J.L., Ledbetter, J.A., Singh, C., and Tepper, M.A. (1992) Science(Wash. DC). 257, 792-795.
16. Turka, L.A., Linsley, P.S., Lin, H., Brady, W., Leiden, J.M., Wei, R.Q., Gibson, M.L., Zheng, X.G., Myrdal, S., Gordon, D., Baily, T., Bolling, S.F., and Thompson, C.B. (1992) Proc. Natl. Acad. Sci. USA. 89, 11102-11105.
17. Lenschow, D.J., Zeng, Y., Thistlethwaite, J.R., Montag, A., Brady, W., Gibson, M.G., Linsley, P.S., and Bluestone, J.A. (1992) Science(Wash. DC). 257, 789-792.
18. Wu, Y., Y. Guo, Y., and Y. Liu, Y. (1993) J. Exp. Med. 178, 1789-1793.
19. Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A. (1988) Science(Wash. DC). 239, 487-491.
20. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156-159.
21. Zhou, T., Bluethmann, H., Zhang, J., Edwards III,C, K., and Mountz, J.D. (1992) J. Exp. Med. 176, 1063-1072.
22. Selvakumar, A., White, P.C., and Dupont, B. (1993) Immunogenetics. 38, 292-295.