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Association of the Putative B-Lymphocyte Surface Molecule B7.3 with a Protein Kinase Activity

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Monoclonal antibody BB1 was previously shown to recognize the CD80 protein as well as a putative B7.3 molecule, both ligands of the important T-cell receptors CD28 and CTLA-4. We report that BB1 co-precipitated from detergent lysates of human B- and pre-B-cell lines a protein kinase activity, as detected by solid phase immunoprecipitation followed by *in vitro* kinase assay. As other monoclonal antibodies to CD80 did not co-precipitate any kinase activity, it seems likely that this protein kinase is associated with the so far poorly characterized putative B7.3 molecule. © 1996 Academic Press, Inc.

Productive stimulation of T-lymphocytes requires simultaneous delivery of at least two signals: The first one is mediated by the antigen-specific receptor of T-cells (TCR), the other, costimulatory signal, results from binding of the T cell surface receptor called CD28 to its ligands CD80 (B7/BB1) and CD86 (B7.2) expressed on the surface of APC (1). In addition to CD28 on the surface of activated T-cells, there is another receptor called CTLA-4 which binds both CD80 and CD86 ligands with affinities much higher than CD28. CTLA-4 appears is, in contrast to CD28 an inhibitory rather than stimulatory receptor (2, 3). At present it is not quite clear what are binding preferences of CD28 and CTLA-4 for CD80 vs. CD86, but a significant point is that CD86 is upregulated much more quickly than CD80 upon activation of B-cells and that CD28 is expressed constitutively on T-cells while CTLA-4 is an activation antigen. All these molecules (CD28, CTLA-4, CD80, CD86) are members of the immunoglobulin structural superfamily (for review see Ref. 4).

Among several monoclonal antibodies to CD80 (5) there is one called BB1 (6), which differs in its specificity from the others. In addition to CD80, it seems to recognize yet another molecule expressed on cells that do not possess CD80 (e.g. thymic epithelial cells, keratinocytes, a subset of activated B-cells) (7). This so far uncharacterized molecule was suggested to be called B7.3 and it also seems to be an alternative ligand of CTLA-4 (7). Therefore, CD80 and the putative B7.3 molecule seem to share an epitope uniquely recognized by the BB1 mAb.

During our studies within the 5th International Workshop on Human Leucocyte Differentiation Antigens we looked for B-cell surface proteins associated with protein kinase activities that might be important in signaling mediated by such receptors. In the present communication we report on our finding that mAb BB1 but no other CD80 mAbs co-precipitated a protein kinase from B-cell line detergent lysates. Some of the present data were published in a preliminary form before (8).

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Abbreviation: mAb-monoclonal antibody.

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MATERIALS AND METHODS

Reagents and cells. Autoradiography films, sodium vanadate, chemicals for SDS PAGE and standards of phospho-amino acids were purchased from Sigma (St. Louis, MO), mol. wt. standards and Sepharose 4B from Pharmacia (Uppsala, Sweden), NP-40 from Fluka (Buchs, Switzerland), [γ-^{32}P]ATP from Amersham Buchler (Braunschweig, Germany), endoglycosidase F and endoglycosidase H from Boehringer (Mannheim, Germany), kinase inhibitors (quercetin, genistein, herbimycin) were kindly provided by Dr. F. Lund-Johansen (Gade Hospital, Bergen, Norway). B cell line Raji was obtained from the collection of the Institute of Haematology and Blood Transfusion (Prague).

Antibodies. MAb BB1 (6) was kindly provided by Dr. E. A. Clark (University of Washington, Seattle, WA), other CD80 mAbs (133, 49, B7-g, L307) as well as all CD19 mAbs were obtained as a part of the B cell panel of the 5th International Workshop on Human Leukocyte Differentiation Antigens (9). Control mAbs MEM-102 (CD48; IgG1) and MEM-28 (CD45; IgG1) were prepared in our laboratory. All mAbs used in this study bound specifically to the B cell line Raji, as tested by indirect cytofluorometry (not shown).

Cell solubilization, fractionation of the lysate, immunoprecipitation in vitro phosphorylation, endoglycosidase treatments and phospho-amino acid analysis. Cells (5×10^7/ml) were lysed for 30 min at 0 °C in a lysis buffer containing 150 mM NaCl, 20 mM Tris-HCl pH 8.2, 1 mM phenylmethylsulfonylfluoride and 1 % NP-40 with or without 5 mM iodoacetamide. Nuclei and other insoluble materials were removed by low speed centrifugation (20 000 $\times$ g, 3 min) and the supernatant was used for further work. Immunoprecipitation using a modification of the solid phase immunoisolation technique with antibodies immobilized on plastic well surface, *in vitro* kinase assay on the immunoprecipitates, phospho-amino acid analysis and SDS PAGE were performed as described before (10, 11). Gel chromatography of the detergent lysate on 3 ml columns of Sepharose 4B was performed as described before (12) and the fractions obtained were used for immunoprecipitation followed by *in vitro* kinase assay. Treatments of radiolabeled immunoprecipitates with endoglycosidase H and endoglycosidase F were also described before (10). To examine the effects of kinase inhibitors, the kinase solution without [γ-^{32}P]ATP was supplemented with the substances tested in the concentrations indicated, added to the immunoprecipitates (formed on the walls of antibody-coated plastic wells) and incubated 30 min on ice. Then [γ-^{32}P]ATP was added and kinase reaction was allowed to proceed at room temperature for 20 min. After washing, the *in vitro* phosphorylated proteins were eluted by the SDS PAGE sample buffer, analyzed by SDS PAGE and detected by autoradiography.

RESULTS

Association of protein kinase activity with an antigen recognized by mAb BB1. In vitro kinase assay on the BB1 immunoprecipitate of Raji cell lysates revealed the presence of a protein kinase activity: two major *in vitro* phosphorylated zones were observed of mol. wt. corresponding to 51 and 61 kDa, irrespective of whether the sample was reduced before electrophoresis or not; a third weaker zone of rather variable intensity (mol. wt. 56 kDa) was also detectable in some experiments (Fig. 1).

Four other CD80 mAbs (133, 49, B7-g, L307) were negative in this assay. Similar results were observed also when pre-B cell line Nalm-6 was used instead of Raji while myeloid cell line HL-60 was negative (not shown). Three out of 20 tested mAbs directed to CD19 (AB1, B43, HD37) immunoprecipitated also a protein kinase activity apparently similar to the BB1-associated one; in this case the pattern of the *in vitro* phosphorylated zones contained in addition also 90 kDa zone, presumably the CD19 glycoprotein itself (Fig. 1).

Characterization of the kinase-containing complexes. The results of phospho-amino acid analysis revealed that the three major zones phosphorylated *in vitro* in the BB1 immunoprecipitate (as well as in the similar CD19 immunoprecipitates) were phosphorylated on threonine and tyrosine residues but not on serine (not shown).

To examine the size of the BB1/protein kinase-containing complexes, Raji cell detergent lysate was subjected to gel chromatography on Sepharose 4B, an extremely porous gel (exclusion limit of 20-40 $\times$ 10^3 kDa) which efficiently separates some very large protein kinase-containing membrane complexes from uncomplexed molecules. The BB1-associated kinase activity was found in the fractions corresponding to relatively small complexes (up to approx. 10^3 kDa) but not in the void volume fractions containing the very large membrane complexes containing Src-family kinases studied previously by us (12) (Fig. 1b). The *in vitro* phosphorylated proteins were relatively stable associated with the immunoprecipitate, as we were not

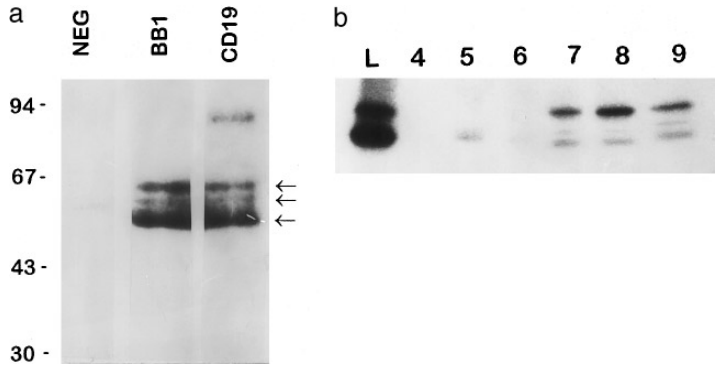

FIG. 1. (a) Immunoprecipitation of the indicated molecules followed by *in vitro* kinase assay. The detergent (1% NP-40) lysate of Raji cells was immunoprecipitated using plastic wells coated by specific mAbs and the immunoprecipitates were subjected to *in vitro* kinase assay. The endogenous proteins phosphorylated by the associated protein kinases were then eluted by a non-reducing sample buffer, analyzed by SDS-PAGE on 10% gel and the radiolabeled proteins were visualized by autoradiography. The mAbs used were MEM-111 (CD54) (NEG.; negative control), BB1, and B43 (CD19). The arrows point to the 61, 56, and 51 kDa phosphoproteins, respectively. The positions of molecular weight standards (kDa) are indicated. (b) Detection of the kinase-containing complexes after fractionation of the NP-40 Raji cell lysate by Sepharose 4B gel chromatography. The fractions obtained were subsequently used for immunoprecipitation on the BB1 mAb followed by *in vitro* kinase assay and autoradiography. Fraction numbers (4 through 9) are indicated at the top; the lane denoted L corresponds to the unfractionated cell lysate. It should be noted that elution maxima of size standards (determined in a separate run) erythrocytes, IgM (900 kDa), IgG (150 kDa), and bromophenol blue were in fractions 4, 6, 8, and 9, respectively. Only the relevant part of the autoradiogram (between 43 and 67 kDa) is shown.

able to release them efficiently from the immunosorbent by 30 min incubation with 1 % NP40 or 1 % CHAPS at 37 °C or with acidic buffer (0.1 M citrate pH 2.9 with 0.1 % NP40). Most of these phosphoproteins were released from the immunoprecipitate by incubation with 0.2 % SDS or by alkaline buffer (0.1 M glycine-NaOH, pH 11.5 with 0.1 % NP40). The phosphoproteins eluted from the immunoprecipitates by either 0.2 % SDS or the alkaline buffer were diluted with the NP40-containing lysis buffer and used for re-precipitation with the BB1 mAb. However, no radiolabeled material was re-precipitated under these conditions (not shown), indicating that the association of the phosphoproteins with the antigen recognized by BB1 mAb was irreversibly disrupted under the elution conditions. The *in vitro* phosphorylated proteins were not sensitive to treatments with endoglycosidase F or endoglycosidase H, indicating that they are not N-glycosylated glycoproteins (Fig. 2).

The BB1-associated protein-kinase activity was strongly inhibited by low concentration of Cu^{2+}, EDTA and staurosporine and partially also by iodoacetamide, H7, quercetin and genistein but not by herbimycin (not shown).

DISCUSSION

MAb BB1 is unique among the anti-CD80 mAbs as it appears to recognize in addition to the CD80 glycoprotein an additional molecule (7). This putative B7.3 molecule sharing an epitope with CD80 may also function as an alternative ligand of the T cell surface receptors CD28 and CTLA-4 (7). In this communication we confirm uniqueness of this mAb as reflected in its ability to co-precipitate a protein kinase activity from detergent lysate of Raji B-cells (and also pre-B cell line Nalm-6, not shown here). As other CD80 mAbs tested do not co-precipitate such a kinase activity, it is reasonable to assume that the kinase activity is not associated with CD80 but with the putative B7.3 molecule recognized by the BB1 mAb. The

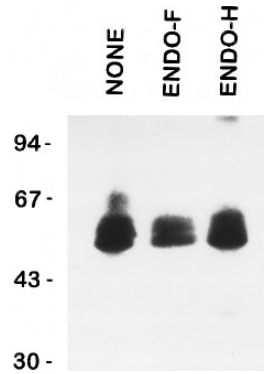

FIG. 2. Insensitivity of the *in vitro* phosphorylated proteins to endoglycosidase treatments. The samples obtained by immunoprecipitation on the BB1 mAb and *in vitro* kinase assay (see Fig. 1a) were treated with endoglycosidase F (ENDO-F), endoglycosidase H (ENDO-H), or mock-treated (NONE), subjected to SDS-PAGE and autoradiography. Positions of molecular weight standards (kDa) are indicated.

nature of the hypothetical B7.3 molecule is unknown. We were unable to detect any specific zone by Western blotting of Raji cell lysates by means of the BB1 mAb; the only (rather weak) specific material immunoprecipitated by BB1 (and also by the other CD80 mAbs) from NP-40 lysate of surface radioiodinated Raji cells was an approx. 65 kDa zone, apparently the CD80 glycoprotein (P. Angelisová, unpublished). Therefore, the putative B7.3 molecule may be expressed at low level and/or is poorly labeled by surface radioiodination. The results of our reprecipitation experiments indicate that none of the *in vitro* phosphorylated proteins is directly recognized by BB1 (i.e. none of them is identical to the putative B7.3 molecule), but rather are noncovalently associated with the BB1-reactive antigen. However, this association must be relatively strong as it is not broken by incubation in nonionic detergents at 37 °C or by low pH. At present it is not clear whether some of the *in vitro* phosphorylated proteins is a self-phosphorylating protein kinase or whether they are protein substrates of an ''invisible'' protein kinase. We were unable to demonstrate by reprecipitation or by Western blotting the presence of the Lck or Lyn protein kinases in the BB1 immunoprecipitates (unpublished date). The phosphorylation on both threonine and tyrosine residues observed in all phosphoproteins found in the BB1 immunoprecipitate may either indicate involvement of two protein kinases with different substrate specificities, or activity of a kinase with an unusual threonine/tyrosine double-specificity.

The last point to be mentioned is the similarity of the *in vitro* phosphoprotein pattern observed in the BB1 and some CD19 immunoprecipitates. Possibly the protein kinase (or a complex of a protein kinase with its substrates) associated with the putative B7.3 molecule is also associated with a subpopulation of CD19 molecules recognized by some but not all anti-CD19 mAbs. CD19 is known to be a component of an important receptor complex on the B-lymphocyte surface which contain protein tyrosine kinases (13). Possible relationship between these CD19-containing and putative B7.3-containing complexes is a matter of further investigation. It is premature to speculate on possible functional importance of the protein kinase(s) associated with the putative B7.3 molecule recognized by the BB1 mAb, but it seems likely that such an association would endow the B7.3 receptor with a signalling capacity.

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