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Immunology

Identification, by protein sequencing and gene transfection, of sgp-60 as the murine homologue of CD48

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ABSTRACT We have recently described a murine lymphocyte protein, provisionally termed sgp-60, which is expressed on virtually all lymphocytes of both T- and B-cell origin. A hamster monoclonal antibody to sgp-60 can inhibit interleukin-2 (IL-2) production, IL-2-receptor expression, and T-cell proliferation, events normally observed after stimulation of T cells with an antibody to the T-cell receptor/CD3 complex or with the lectin concanavalin A. Our previous studies did not reveal the molecular nature of the sgp-60 antigen. Purification of sgp-60 and protein sequencing demonstrate that sgp-60 is identical to the CD48 antigen, a ligand for the CD2 antigen, which is also called Blast-1 in humans, BCM1 in mice, and OX-45 in rats. The identity of sgp-60 and CD48 was independently confirmed in gene transfection experiments. The anti-sgp-60 monoclonal antibody was selectively reactive with COS-7 cells transfected with a BCM1 cDNA clone but not with K^b-transfected controls. The results of the present report, together with our previous functional studies, may have implications for the role of CD2 and CD48 in murine T-cell activation.

Antigen-specific $CD4^+$ T inducer cells are critically involved in the generation of delayed-type hypersensitivity, antibody, and cytotoxic responses. Normally, $CD4^+$ T cells are in a resting state. Most immune responses therefore depend on the prior activation of $CD4^+$ T lymphocytes, their rapid proliferative expansion, and the lymphokines which they secrete (1).

The activation of $CD4^+$ T cells depends critically on cognate interactions of the T cell with an antigen-presenting cell (APC). Physiologic activation is imparted by a heterodimeric T-cell receptor (TCR) with specificity for antigenic peptides complexed to major histocompatibility complex (MHC) proteins. The TCR is associated with a set of nonvariable proteins, collectively termed CD3 (T3) (2). Besides the polypeptides forming the TCR/CD3 complex, a number of molecules participate in the T-cell activation process. Some function as cell–cell interaction molecules and some as signal-transducing elements, and some can serve both functions (2).

We have recently described a murine glycoprotein, provisionally termed sgp-60, which is expressed on virtually all lymphocytes of both T- and B-cell origin (3–5). Antibody inhibition studies suggest a functional role for sgp-60 in $CD4^+$ T lymphocytes: addition of the anti-sgp-60 monoclonal antibody (mAb) 5-8A10 or of its Fab fragment significantly inhibits the activation of purified T cells by the lectin Con A or by a mitogenic anti-CD3 mAb (3, 4). Proliferation, interleukin 2 (IL-2) production, IL-2 receptor expression, and generation of second messengers are all affected by the anti-sgp-60 mAb (3, 4, 6). Only a few antibodies affect T-cell function. Our experiments therefore suggest a role for sgp-60 in T-cell activation.

Our previous studies did not reveal the molecular identity of the sgp-60 antigen. In the present report, we close this gap. Partial amino acid sequence of the sgp-60 antigen and gene transfection experiments demonstrate that sgp-60 is identical to the CD48 antigen, which is also called Blast-1 in humans (7, 8), BCM1 in mice (9), and OX-45 in rats (10, 11).

MATERIALS AND METHODS

Reagents. All reagents were obtained from Sigma unless otherwise noted.

Antibodies and Cell Lines. The following mAbs were used in this study: 5-8A10 = anti-sgp-60 (3, 4); B8-24-3 = anti-K^b (12); 16-10A1 = anti-mB7 (13), M5/114 = anti-I-$A^{b,d,q}$, anti-I-$E^{d,k}$ (14), and HM48-1 = anti-CD48 (28). The mAb-secreting hybridomas listed above and the T-cell lymphoma EL-4 (15) were passaged in Dulbecco's modified Eagle's medium containing 4.5 g of glucose per liter (Irvine Scientific), supplemented with 10% fetal calf serum, L-glutamine, and antibiotics. COS-7 cells (16) were passaged in RPMI 1640 medium (Irvine Scientific) supplemented with 10% fetal calf serum, L-glutamine, and antibiotics.

Cell Fractionations. Mouse T cells were purified as previously described (3, 4). Briefly, splenocytes from BALB/c mice were depleted of erythrocytes by treatment with Tris/NH_4Cl. T cells were enriched by nylon wool fractionation (17) and further purified by two-fold treatment with the anti-MHC class II mAb M5/114 and rabbit complement (Cedarlane Laboratories, Hornby, ON).

Preparative-Scale Purification of sgp-60. EL-4 lymphoma cells were grown in bulk culture, harvested, and washed with phosphate-buffered saline (PBS). The pellet, containing about 30 g of total protein, was resuspended in 30 ml of lysis buffer [10 mM triethanolamine, pH 7.8 (adjusted with HCl)/150 mM NaCl/1% Nonidet P-40/1 mM phenylmethylsulfonyl fluoride (PMSF)/10 mM iodoacetate containing the protease inhibitors leupeptin, pepstatin, chymostatin, antipain, and N^{α}-*p*-tosyl-L-lysine chloromethyl ketone at 1 μg/ml each] and stored at −80°C until use. The crude extract was diluted with 270 ml of the same buffer, but with 1% *n*-octyl β-D-glucopyranoside (octyl glucoside) instead of Nonidet P-40 and stirred for 1 hr at room temperature and subsequently overnight at 4°C. The lysate was clarified by centrifugations at 1500 × *g* for 15 min and then at 100,000 × *g* for 90 min, both at 4°C. The supernatant was passed over sequential columns of staphylococcal protein A-Sepharose, control mouse immunoglobulin-coupled CNBr-activated Sepharose 4B, and mAb 5-8A10-coupled CNBr-activated Sepharose 4B, all at 4°C and at a flow rate of 0.5 ml/min. The last column was washed with 5 column-bed vol of 10 mM triethanolamine, pH

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Abbreviations: APC, antigen-presenting cell; mAb, monoclonal antibody; TCR, T-cell receptor.

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7.8/250 mM NaCl/1% octyl glucoside/1 mM PMSF/10 mM iodoacetate, then washed with 10 vol of the same buffer at pH 10, and finally with 5 vol at pH 7.8. The elution was carried out with 3 M NaSCN in 10 mM triethanolamine, pH 7.8/150 mM NaCl/1% octyl glucoside/1 mM PMSF/10 mM iodoacetate. Fractions (4 ml) were collected and frozen at −80°C. Aliquots (40 μl) from each fraction were analyzed by $NaDodSO_4$/14% PAGE (18). Proteins were detected by silver staining (19). Fractions containing material of the expected molecular mass were pooled, concentrated in a Centricon 30 microconcentrator (Amicon), and dialyzed in the same unit against 10 mM triethanolamine, pH 7.8/150 mM NaCl/1 mM PMSF/10 mM iodoacetate.

The material was further purified by preparative $NaDodSO_4$/PAGE according to Thomas and Kornberg (18), using 1 mm-thick 10% gels under nonreducing conditions. Proteins were electroblotted with a commercial apparatus (Hoefer) onto nitrocellulose membrane (pore size 0.45 μm, Schleicher & Schuell) as described (20).

Trypsin Digestion, HPLC Separation, and Protein Microsequencing. After electrophoretic transfer to nitrocellulose, protein was visualized by staining with Ponceau S (21). The appropriate protein band was excised, destained, and subjected to *in situ* digestion with trypsin (22), omitting the NaOH wash. Peptides were separated by narrow-bore high-performance liquid chromatography using a Vydac (Hesperia, CA) C_{18} 2.1 × 150-mm reverse-phase column on a Hewlett–Packard 1090 HPLC with a 1040 diode array detector. Fractions from this chromatogram were chosen based on differential UV absorbance at 210 nm, 277 nm, and 292 nm, peak symmetry, and resolution. Selected fractions were submitted to automated Edman degradation on an Applied Biosystems 477A protein sequencer using a microcartridge and cycles optimized for a 30-min cycle time. Details of strategies for the selection of peptide fractions and their microsequencing have been previously described (23).

Clone Isolation and DEAE-dextran Transfection. The complete coding region of BCM1 (9) was amplified by polymerase chain reaction (PCR) from an A20 B cell cDNA library into the pCDM8 vector (24). The sense primer (5′-GCTAAAGCT-TCTCGAGCCGCCACCATGTGCTTCATAAAACAGG-GAT-3′) consisted of an oligonucleotide corresponding to nucleotides 51–72 of BCM1 plus a strong translational start signal and restriction sites for *Hin*dIII and *Xho* I. The antisense primer (5′-CGTAAAGCTTCTCGAGTCTA-GAGTTCTTGTCAGGTTAACAG-3′) corresponds to nucleotides 783–762 of BCM1 and includes the translational stop signal plus sites for *Xba* I, *Xho* I, and *Hin*dIII. PCR was performed with *Taq* DNA polymerase and the GeneAmp kit (Perkin–Elmer/Cetus) according to the manufacturer's instructions. Thermal cycling conditions were 94°C for 1 min, 53°C for 1 min, and 72°C for 1 min, for 30 cycles. The 0.8-kb product was digested with *Hin*dIII, ligated into the *Hin*dIII site of the pcDNAI expression vector, and used to transform *Escherichia coli* DH10B/p3 (GIBCO/BRL). Plasmid DNA was prepared from individual colonies by the alkaline lysis procedure. The orientation of the cDNA insert was determined by digestion with *Xba* I.

Plasmid DNA with a 0.8-kb insert was individually transfected into COS-7 cells, at 50–60% confluency, in 100-mm culture dishes, by using the DEAE-dextran method followed by osmotic shock with 10% (vol/vol) dimethyl sulfoxide (25). Cells were incubated for 48–72 hr to allow transient expression.

DNA Sequencing. cDNA inserts were sequenced by using dye-labeled primers and *Taq* polymerase (Applied Biosystems). Sequencing reactions were analyzed on an Applied Biosystems (model 373) automated fluorescent sequencer.

Radiolabeling and Immunoprecipitations. Cell surface proteins were iodinated in a lactoperoxidase-catalyzed reaction (26) with 1 mCi (37 MBq) of $Na^{125}I$ per 3 × 10^7 cells. Immunoprecipitations were carried out essentially as previously described (4). Immunoprecipitates were analyzed on $NaDodSO_4$/14% PAGE gels (18). Autoradiograms were exposed with an intensifying screen for 3 days at −80°C.

Indirect Immunofluorescence and Flow Fluorocytometry. The expression of cell surface antigens by transfected COS-7 cells was studied by immunofluorescence as previously described (4, 27) by using mAb culture supernatants and, as a second step reagent, fluorescein isothiocyanate (FITC) goat anti-mouse IgG (Cappel Laboratories), crossreactive with hamster IgG. Samples were analyzed on a FACScan (Becton Dickinson).

RESULTS

Purification and Microsequencing of sgp-60. sgp-60 was purified from EL-4 lymphoma cells. We had previously shown that this cell line expresses sgp-60 (4). Purification conditions were determined at an analytical scale with ^{125}I-labeled cell lysates. Initial experiments demonstrated that mAb 5-8A10-coupled Sepharose could bind and immunoprecipitate the sgp-60 antigen. A comparative analysis of different elution conditions subsequently revealed that sgp-60 remained largely bound to mAb 5-8A10-coupled Sepharose beads at pH 11 or pH 3. In contrast, the protein could be efficiently eluted at higher concentrations (>1 M) of NaSCN at pH 7.8 (data not shown). This latter method was therefore chosen for large-scale purification.

For this purpose, a lysate of EL-4 cells was passed over a series of nonspecific columns and finally over a mAb 5-8A10 affinity column as detailed in *Materials and Methods*. sgp-60 was eluted in fractions from this latter column by using a buffer containing 3 M NaSCN. Aliquots from each fraction were analyzed by $NaDodSO_4$/PAGE and silver staining. Fractions containing sgp-60 were pooled, concentrated, and fractionated on a preparative $NaDodSO_4$/polyacrylamide gel. Subsequently, the proteins were electroblotted to nitrocellulose. A band migrating with an apparent molecular mass of 65 kDa was cut out and subjected to tryptic digestion, reverse phase-HPLC separation, and microsequencing. Two peptide sequences were obtained. The sequences are shown in Fig. 1. A computer search revealed that both sequences matched with the previously published sequence of the BCM1 antigen, the murine homologue of CD48. In particular, the first peptide sequence corresponds to residues 62–76 of BCM1, and the second sgp-60 peptide corresponds to residues 52–58. These results strongly suggested that the sgp-60 and BCM1 antigens were identical.

Isolation of cDNA Clones Encoding BCM1: The Anti-sgp-60 mAb Recognizes the Transiently Expressed Gene Product. This result was unexpected because previous $NaDodSO_4$/PAGE analysis had suggested a molecular mass for sgp-60 of approximately 60 kDa (4). In contrast, the published molec-

CYCLE	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
PEPTIDE #1	Val	Tyr	Leu	Glu	Glu	Asn	Asn	Gly	Ala	Leu	(His)	(Ile)	(Ser)	Asn	Val	---
PEPTIDE #2	Thr	Ile	Phe	Glu	Ser	Glu	Phe	---								

FIG. 1. Partial amino acid sequence of the sgp-60 antigen. sgp-60 was purified by immunoaffinity chromatography and subsequent preparative $NaDodSO_4$/PAGE. The purified sgp-60 protein was subjected to tryptic digestion and reverse phase-HPLC separation. Microsequencing yielded the two peptide sequences shown. Parentheses indicate a residue identified with low confidence.

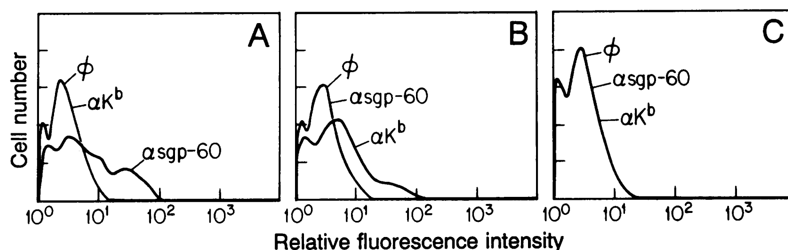

FIG. 2. Staining of BCM1 transfectants by the anti-sgp-60 mAb 5-8A10. COS-7 cells were transfected with pcBCM1-1 cDNA (*A*) or the K^b-encoding plasmid pcDL-SRα296 (*B*) or were mock transfected (*C*). After 72 hr, cells were harvested and analyzed by indirect immunofluorescence and flow cytometry. In the first step, cells were incubated as indicated with medium (ϕ), anti-sgp-60 mAb 5-8A10 (αsgp-60), or anti-K^b mAb B8-24-3 (αK^b). The second-step reagent was a fluorescein isothiocyanate (FITC)-labeled goat $(Fab')_2$ anti-mouse IgG antibody (Tago) that crossreacts with hamster IgG. Samples were analyzed on a FACScan (Becton Dickinson); 5000 cells were analyzed per sample.

ular mass of CD48 is approximately 45–50 kDa (9). We therefore tried to independently determine whether the sgp-60 and BCM1 antigens were identical. For this purpose, BCM1 cDNA was amplified from a cDNA library by PCR. The PCR product was ligated into the pcDNAI expression vector. Plasmid DNA was obtained from individual colonies. All of the clones studied (a total of 12) contained a cDNA insert of 0.8 kb. Two clones were selected for further analysis. One of the two clones was sequenced by automated fluorescent sequencing, using dye-labeled primers and *Taq* polymerase. The nucleotide sequence of this BCM1 clone, pcBCM1-1, was identical to the published BCM1 sequence except for a single nucleotide change, predicting an amino acid change at position 68 from Asn to Asp. Both clones were used in gene transfection experiments. For this purpose, COS-7 cells were transfected by using the DEAE-dextran method. Cells were incubated for 72 hr to allow transient expression. After the incubation period, cell surface expression of antigens was studied by indirect immunofluorescence and flow fluorocytometry. Fig. 2 shows a representative experiment using one of the BCM1 cDNA clones, pcBCM1-1. As shown in Fig. 2*A*, the anti-sgp-60 mAb 5-8A10 reacts with the product of this BCM1 clone in transfected COS-7 cells. Two findings support the specificity of this result. First, a control mAb, B8-24-3 (anti-K^b), does not react with the BCM1 transfected cell (Fig. 2*A*). This latter mAb recognizes the product of a K^b-encoding plasmid, pcDL-SRα296 (Fig. 2*B*). Second, the anti-sgp-60 mAb 5-8A10 does not react with K^b-transfected (Fig. 2*B*) or mock-transfected (Fig. 2*C*) COS-7 cells. Taken together, these data convincingly demonstrate that the anti-sgp-60 mAb recognizes the BCM1 antigen on the surface of BCM1-transfected COS-7 cells. Gene transfection experiments using a second BCM1 clone yielded a qualitatively similar result (data not shown).

Immunoprecipitation of BCM1 from Labeled COS-7 Cell Transfectants. As mentioned above, previous analyses suggested a difference in the apparent molecular masses of sgp-60 and BCM1 (4, 9). It was therefore of interest to determine the apparent molecular mass of the BCM1 product in transfected COS-7 cells after immunoprecipitation with the anti-sgp-60/BCM1 mAb 5-8A10. For this purpose, COS-7 cells were transfected with the BCM1-encoding plasmid pcBCM1-1. Three days after the transfection, cells were labeled with ^{125}I in lactoperoxidase-catalyzed reactions. Immunoprecipitations were carried out with protein A-Sepharose. The immunoprecipitates were eluted by incubation with

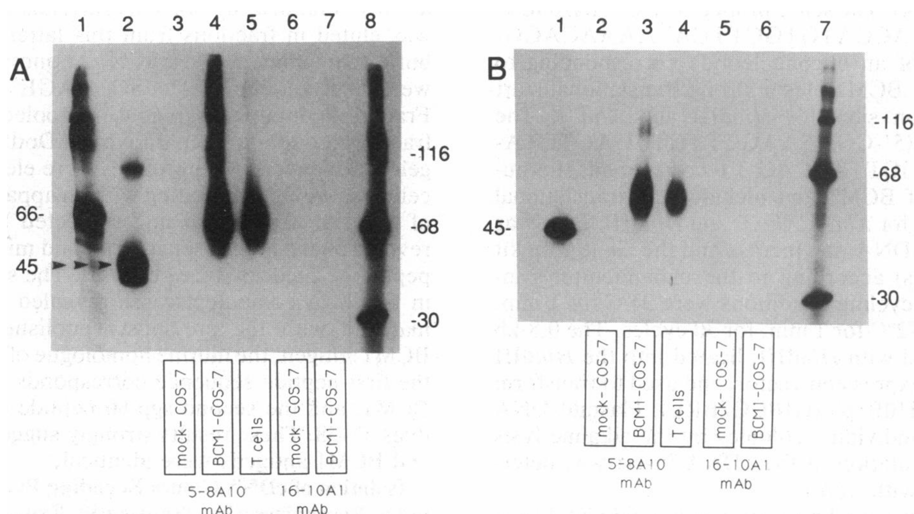

FIG. 3. Immunoprecipitation of the BCM1 antigen by anti-sgp-60 mAb: $NaDodSO_4$/PAGE analysis under nonreducing and reducing conditions. (*A*) Analysis under nonreducing conditions. BCM1-transfected COS-7 cells (lanes 4 and 7), mock-transfected COS-7 cells (lanes 3 and 6), or splenic T cells (lane 5) were surface iodinated in lactoperoxidase-catalyzed reactions. Immunoprecipitations were performed as described, using either mAb 5-8A10 (anti-sgp-60; lanes 3–5) or mAb 16-10A1 (anti-mB7; lanes 6 and 7). The following molecular mass markers were used in this experiment: ^{14}C-labeled bovine serum albumin (Sigma; 66 kDa; lane 1); ^{14}C-labeled ovalbumin (Sigma; 45 kDa; lane 2); and mixture of ^{125}I-labeled molecular mass markers (New England Nuclear; molecular masses indicated; lane 8). (*B*) Analysis under reducing conditions. BCM1-transfected COS-7 cells (lanes 3 and 6), mock-transfected COS-7 cells (lanes 2 and 5), or splenic T cells (lane 4) were surface iodinated in lactoperoxidase-catalyzed reactions and lysed in IPB. Immunoprecipitations were performed as described using either mAb 5-8A10 (anti-sgp-60; lanes 2–4) or mAb 16-10A1 (anti-mB7; lanes 5 and 6). The following molecular mass markers were used in this experiment: ^{14}C-labeled ovalbumin (Sigma; 45 kDa; lane 1); mixture of ^{125}I-labeled molecular mass markers (New England Nuclear; molecular masses indicated; lane 7). All immunoprecipitates were analyzed on 14% polyacrylamide gels. All molecular masses are indicated in kDa.

$NaDodSO_4$ in the presence or absence of the reducing agent 2-mercaptoethanol. The polypeptides were fractionated on $NaDodSO_4$/polyacrylamide gels in the procedure of Thomas and Kornberg (18). Representative experiments are shown in Fig. 3. The mAb 5-8A10 immunoprecipitated a prominent band from BCM1-transfected COS-7 cells. The immunoprecipitated polypeptide migrates with an apparent molecular mass of 65 kDa under nonreducing conditions (Fig. 3*A*, lane 4) and with an apparent molecular mass of 60 kDa under reducing conditions (Fig. 3*B*, lane 3). Polypeptides with similar molecular masses are immunoprecipitated from T-cell lysates (Fig. 3*A*, lane 5; Fig. 3*B*, lane 4). The above immunoprecipitations are specific, as the 60- and 65-kDa bands were not observed in mAb 5-8A10 immunoprecipitates from lysates derived from mock-transfected lysates (Fig. 3*A*, lane 3; Fig. 3*B*, lane 2). Furthermore, the 60- to 65-kDa bands were not immunoprecipitated by a control hamster mAb of irrelevant specificity (Fig. 3*A*, lanes 6 and 7; Fig. 3*B*, lanes 5 and 6). This latter mAb, the anti-mB7 mAb 16-10A1, immunoprecipitates an unrelated protein of similar mass from other cells (data not shown and ref. 13). Taken together, the results of the immunoprecipitation experiments further demonstrate that mAb 5-8A10 reacts with the BCM1 protein. Our data also indicate that the BCM1 molecules from normal T cells and from transfected COS-7 cells migrate with similar apparent molecular mass when analyzed by $NaDodSO_4$/PAGE.

DISCUSSION

In the present report we have characterized the glycoprotein defined by the anti-sgp-60 mAb. Microsequencing of purified and enzymatically cleaved sgp-60 yielded two internal peptide sequences. These sequences match with the previously published sequence for the murine CD48 antigen, BCM1. The identity of sgp-60 and BCM1 was independently confirmed in gene transfection experiments. The anti-sgp-60 mAb 5-8A10 reacts with BCM1-transfected COS-7 cells: it specifically stains intact transfected cells and immunoprecipitates BCM1 molecules from detergent lysates of these cells.

The identity of sgp-60 and CD48 was unexpected because previous $NaDodSO_4$/PAGE analysis had suggested a molecular mass for sgp-60 of approximately 60 kDa, while the published molecular mass of BCM1 is approximately 45–50 kDa (9). mAb 5-8A10 immunoprecipitates, from both T cells and BCM1-transfected cells, a protein which migrates with an apparent molecular mass of 60–65 kDa in our $NaDodSO_4$/PAGE system. It remains to be determined whether the differences in migration pattern are due to a technical difference—e.g., in the gel systems used. Comparative immunoprecipitations with mAb 5-8A10 and a more recently described anti-CD48 mAb (28) are necessary to resolve this issue.

The identity of sgp-60 and BCM1 may have implications for our understanding of T-cell function. We have previously shown that mAb 5-8A10 inhibits the activation of $CD4^+$ T cells through the TCR/CD3 complex (3, 4, 6). mAb 5-8A10 can inhibit T-cell activation directly, as inhibitory effects can be observed in the absence of APCs, when purified T cells are stimulated in the presence of phorbol myristate acetate as a costimulatory signal (4). Kato and coworkers have recently shown that CD48 is the ligand of CD2 (28). Our own previous studies (4) as well as the results by Kato *et al.* (28) show that CD48 is widely expressed on both T and B lymphocytes of mice. These findings are consistent with previous observations by Yagita *et al.* (29), who have shown that the murine CD2 antigen also is expressed on both T and B cells. Thus, our findings in the present report, together with our previous functional studies using mAb 5-8A10, raise the possibility that the CD2 molecule functions not only as a receptor but also as a ligand in murine T-cell activation.

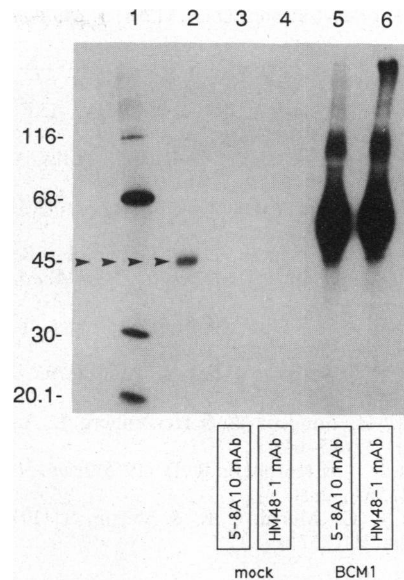

FIG. 4. Comparative immunoprecipitation of CD48 using the mAbs 5-8A10 and HM48-1. BCM1-transfected COS-7 cells (lanes 5 and 6) or mock-transfected COS-7 cells (lanes 3 and 4) were surface iodinated in lactoperoxidase-catalyzed reactions. Immunoprecipitations were performed using either mAb 5-8A10 (lanes 3 and 5) or mAb HM48-1 (lanes 4 and 6). The following molecular mass markers were used in this experiment: mixture of ^{125}I-labeled molecular mass markers (New England Nuclear; molecular masses indicated in kDa; lane 1); ^{14}C-labeled ovalbumin (Sigma; 45 kDa; lane 2). All immunoprecipitates were analyzed on 14% polyacrylamide gels under reducing conditions.

Note Added in Proof. Since submission of this manuscript we have carried out comparative immunoprecipitations with the mAb 5-8A10 and the more recently described anti-CD48 mAb HM48-1 (28), kindly provided by Hideo Yagita (Juntendo University, Tokyo), to determine the basis for the observed differences in migration pattern. As shown in Fig. 4, both antibodies immunoprecipitate an antigen which migrates with approximately identical molecular mass when analyzed by the $NaDodSO_4$/PAGE system used in our laboratory. Thus, we have resolved the apparent discrepancy in molecular mass. The remarkable difference in migration pattern of CD48 is likely due to differences in the gel systems used.

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