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Authors

Bell, GM
Seaman, WE
Niemi, EC
et al.

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The OX-44 Molecule Couples to Signaling Pathways and Is Associated with CD2 on Rat T Lymphocytes and a Natural Killer Cell Line

By Gregory M. Bell, William E. Seaman, Eréne C. Niemi, and John B. Imboden

From the Arthritis/Immunology Section, Veterans Affairs Medical Center and University of California, San Francisco, California 94121

Summary

The MRC OX-44 molecule, which is expressed on all peripheral leukocytes, identifies the subset of thymocytes capable of proliferating in response to alloantigens and lectins (Paterson, D. J., J. R. Green, W. A. Jefferies, M. Puklavec, and A. F. Williams. 1987. *J. Exp. Med.* 165:1). When we isolated monoclonal antibodies (mAbs) on the basis of their ability to activate the phosphatidylinositol signaling pathway in RNK-16 cells (a rat leukemia line with natural killer activity), three of the resulting mAbs recognized the OX-44 molecule. Addition of these mAbs to RNK-16 elicits protein tyrosine phosphorylation, generates inositol phosphates, and increases the concentration of cytoplasmic free calcium. These responses require the addition of intact mAb and are not observed with $F(ab')_2$ fragments. One of these mAbs (7D2) is mitogenic for freshly isolated rat splenic T cells and synergizes with a mAb to the T cell antigen receptor in this activation. A 50–60-kD glycoprotein coprecipitates with the OX-44 molecule from RNK-16 cells and rat splenic T cells. Peptide mapping and reprecipitation studies indicate that the coprecipitating molecule is CD2. Thus, the OX-44 molecule can couple to multiple signaling pathways and associates with CD2 on both RNK-16 and rat T cells.

The MRC OX-44 molecule is expressed on all peripheral T cells but only 10% of thymocytes (1). Among thymocytes, only those that express the OX-44 molecule proliferate in response to alloantigens or mitogenic lectins (1, 2). The OX-44 molecule, therefore, is a phenotypic marker for functionally mature thymocytes and may have a role in regulating the responses of thymocytes and peripheral T cells to a variety of mitogenic stimuli. The structure of the OX-44 molecule and its human homologue, CD53, supports this possibility (3–6). The deduced amino acid sequences indicate that the OX-44 molecule and CD53 are members of a newly recognized family of molecules whose distinctive topology is characterized by the presence of four transmembrane spanning domains (3–6). The putative transmembrane and cytoplasmic domains of the OX-44 molecule and CD53 are highly conserved (92% amino acid identity) suggesting that these regions are critical for the function of these molecules (5, 6). These features led Bellacosa et al. (5) to suggest that the OX-44 molecule may have a role in signal transduction. Herein, we provide evidence in support of that hypothesis.

In an effort to identify cell surface molecules capable of stimulating the phosphatidylinositol (PI)[1] signaling pathway in RNK-16 cells (a rat leukemia line with NK cell activity), we screened for mAbs that stimulate the generation of inositol phosphates (InsPs) in RNK-16 cells. This procedure led to the isolation of mAbs directed against only three cell surface structures: CD2, gp42, and, as described here, the OX-44 molecule. Perturbation of the OX-44 molecule not only stimulates a substantial InsP response in RNK-16 cells, but also leads to the appearance of tyrosine-phosphorylated proteins. These responses require the addition of intact mAbs and are not elicited by $F(ab')_2$ fragments. One of the mAbs recognizing the OX-44 molecule (7D2) is mitogenic for freshly isolated splenic T cells, and it augments TCR-mediated proliferation. When RNK-16 cells or freshly isolated rat T cells are solubilized in a buffer containing 3-[(3-cholamidopropyl)-dimethyl ammonio]-1-propane sulfonate (CHAPS),

[1] *Abbreviations used in this paper:* $[Ca^{2+}]_i$, intracellular free calcium; GPI, glycosylphosphatidylinositol; InsP, inositol phosphate; PI, phosphatidylinositol.

a 50–60-kD glycoprotein coimmunoprecipitates with the OX-44 molecule. Peptide mapping and reimmunoprecipitation studies establish that the coprecipitating molecule is CD2. Thus, the OX-44 molecule can couple to signaling pathways and appears to be noncovalently associated with CD2. These properties suggest that the OX-44 molecule may play an important role in the activation of T cells and NK cells.

Materials and Methods

Cells and Cell Culture. RNK-16 cells were adapted for in vitro growth and passaged as described (7). To obtain splenic T cells from Fisher 344 rats (Simonsen Labs, Gilroy, CA), we passed spleens through a no. 40 mesh (Small Parts Inc., Miami, FL). The resulting cell suspension was then washed three times in RPMI supplemented with 10% FCS and resuspended in 5 ml of medium. The cell suspension was centrifuged through 5 ml of Ficoll-Hypaque at 500 *g* for 45 min. Mononuclear cells were harvested from the interface and incubated in a nylon wool column at 37°C for 30 min. Cells eluted from the column were collected and contained ~90% T cells (by flow cytometric analysis after staining with mAb R73).

Antibodies. mAbs OX-18 (IgG1; anti-rat MHC class I antigen), OX-54 (IgG1; anti-rat CD2), OX-55 (IgG1; anti-rat CD2), and OX-44 (IgG1) were obtained from Bioproducts for Science, Inc. (Indianapolis, IN). mAbs OX-34 (IgG2a; anti-rat CD2), OX-8 (IgG1; anti-rat CD8), and R73 (IgG1, anti-rat TCR) were kindly provided by Drs. Alan Williams (University of Oxford) and T. Hunig (Universitate Munchen). mAbs were purified by protein A chromatography. Goat antiserum against mouse Ig and fluorescein-conjugated goat anti-mouse Ig was obtained from Cappel Laboratories (Malvern, PA). For flow cytometry, mAbs were either identified by a second-step antibody or directly conjugated with FITC according to published methods (8). $F(ab')_2$ fragments of mAb 7D2 were prepared by digesting 1 mg of the mAb in 1.0 ml of 0.1 M sodium citrate buffer (pH 3.6) for 1 h at 37°C with 25 μg of pepsin. Remaining intact mAb was removed by protein A chromatography yielding highly purified fragments as assessed by SDS-PAGE.

Generation of mAbs 7D2, 2D1, and 6E2. BALB/c mice were immunized by an intraperitoneal injection of 10^7 RNK-16 cells in CFA. 3 d after an intravenous boost with RNK-16 cells, spleen cells were fused to NS-1 myeloma cells as described (9). Supernatants from the resulting hybridomas were tested initially for binding to RNK-167, as assessed by flow cytometric analysis after staining with a fluorescein-conjugated goat antiserum to mouse Ig. 169 supernatants containing binding mAbs were then tested for the ability to stimulate [^{3}H]InsPs when added to [^{3}H]inositol-labeled RNK-16. Hybridomas that tested positive in this assay were promptly subcloned, and the subclones were again screened for [^{3}H]InsPs generation. This procedure led to the isolation of 7D2 (IgG1), 2D1 (IgG3), and 6E2 (IgG1). These mAbs were purified by protein A chromatography.

Measurement of InsPs and Intracellular Free Calcium ($Ca^{2+}]_i$). InsPs were resolved and quantified as we have described (7), by labeling cells with [^{3}H]inositol (20 μCi/ml) for 3 h before stimulation by mAb. At intervals after stimulation, aliquots of cells (5×10^6) were rapidly pelleted and then lysed in ice-cold 10% TCA. After removal of insoluble material, the supernatant was extensively extracted with diethyl ether. Labeled InsPs were resolved by sequential elution using anion exchange chromatography with Dowex-1X8 (100–200 mesh) in formate form, as described (7). $[Ca^{2+}]_i$ was quantified by monitoring fluorescence of cells loaded with the calcium-sensitive fluor, Indo-1 (Molecular Probes, Eugene, OR), as we have described (7).

Antiphosphotyrosine Immunoblotting. RNK-16 cells were suspended at 10^7 cells/ml in complete medium (RPMI, 10% FCS, L-glutamine [0.3 mg/ml], penicillin K [100 U/ml], and streptomycin sulfate [0.1 mg/ml]) at 37°C, stimulated with mAb at 1 μg/10^6 cells for 2 min, immediately pelleted, then resuspended for 30 min on ice in lysis buffer containing 1% NP-40, 50 mM NaCl, 10 mM Tris (pH 7.4), 50 mM NaF, 30 mM Na pyrophosphate, 0.1 mM $NaVO_4$, 5 mM EDTA, and 1 mM PMSF. Postnuclear supernatants were mixed 3:1 with 4× Laemmli reducing sample buffer, boiled for 2 min, separated by SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) paper. The blot was blocked with 1% gelatin in Tris-buffered saline (TBS; 10 mM Tris, pH 8.0, and 150 mM NaCl) and blotted overnight at 4°C at 1 μg/ml with antiphosphotyrosine mAb (IgG2b; Upstate Biologicals, Inc., Lake Placid, NY), in TBS with 0.05% Tween (TBST). The blot was washed four times in TBST and incubated with a peroxidase-conjugated goat anti-mouse antiserum (Boehringer Mannheim Corp., Indianapolis, IN), that recognizes murine antibodies of the IgG2b subclass, at a 1:500 dilution for 1 h at room temperature. The blot was washed four times in TBST and then developed using 4-chloro-1-naphthol (Bio-Rad Laboratories, Richmond, CA) according to manufacturer's instructions.

Cytotoxicity and Proliferation Assays. Cytotoxicity assays were performed as described (7). For proliferation assays, 2×10^5 nylon wool–nonadherent spleen cells were cultured in 200 μl of complete medium in 96-well microtiter plates for 48 h as described (10). mAbs were added to a final concentration of 10 μg/ml. 6 h before harvesting, each well was pulsed with 1 μCi of [^{3}H]thymidine. Cells were harvested and [^{3}H]thymidine incorporation was detected as described (10).

Surface Labeling, Immunoprecipitation, and SDS-PAGE. 4×10^7 RNK-16 cells were surface labeled with 1 mCi ^{125}I by the glucose oxidase method and were lysed on ice in buffers containing 20 mM TRIS (pH 7.5), 150 mM NaCl, 1 mM PMSF, 20 mM iodoacetamide, and either 10 mM CHAPS or 1% NP-40. Postnuclear supernatants were precleared with preformed complexes of Pansorbin (Calbiochem-Behring Corp., La Jolla, CA) and rabbit anti-mouse IgG antiserum before immunoprecipitation with specific mAb adsorbed to Pansorbin. Immune complexes were then washed three times in lysis buffer containing either 2 mM CHAPS or 0.2% NP-40, and the antigens were eluted with 50 μl of Endo-F sample buffer (0.1% SDS, 50 mM EDTA, 1% 2-ME, and 0.1 M phosphate buffer [pH 6.1]). 0.2 U Endo-F (Calbiochem-Behring Corp.) was added to appropriate aliquots and incubated overnight at 37°C. Alternatively, immunoprecipitates were treated with *N*-glycanase (Genzyme, Boston, MA) according to manufacturer's instructions. An equal volume of twofold concentrated Laemmli sample buffer was added, and the samples were analyzed under reducing conditions by SDS-PAGE, followed by autoradiography. For reprecipitation studies, RNK-16 cells were first surface labeled and lysed in a buffer containing CHAPS. The immune complex was then washed once in 2 mM CHAPS and then resuspended in 150 μl lysis buffer containing 1% NP-40 and allowed to stand at room temperature for 30 min. The immune complex was then pelleted and the supernatant harvested. The immune complex was washed once more and then eluted in Laemmli sample buffer. The NP-40-containing supernatant was subjected to reimmunoprecipitation by specific mAbs. Samples were analyzed under reducing conditions on 10% polyacrylamide gel by SDS-PAGE and autoradiography.

Peptide Mapping. Immunoprecipitations from ^{125}I surface-labeled RNK-16 cells using mAb 2D1 (which recognizes the OX44

molecule), OX-34 (which recognizes rat CD2), and OX-18 (which recognizes rat MHC class I molecules) were carried out as described above. Gel slices from Endo F–treated samples containing class I molecules, directly immunoprecipitated CD2, or the 42-kD protein that coprecipitated with the OX-44 molecule were rehydrated and treated with V8 protease (Calbiochem-Behring Corp.) according to the technique of Cleveland et al. (11). Samples were analyzed on 15% polyacrylamide gels by SDS-PAGE and autoradiography.

Results

Isolation of mAbs that Stimulate PI Turnover in RNK-16 Cells. To identify potential signal-transducing molecules on rat lymphocytes, we generated hybridomas from the spleens of BALB/c mice that had been immunized with RNK-16 cells. The resulting hybridoma supernatants were tested initially for binding to RNK-16. 93 supernatants containing binding mAbs were screened for InsPs production using a goat anti–mouse Ig antiserum as a crosslinking agent. Subcloning of the hybridomas that tested positive led to the isolation of 11 mAbs with agonist properties. An additional 76 supernatants were screened in the absence of a second-step antiserum; this procedure led to the isolation of only one mAb (designated 7D2). Of the 12 mAbs selected, three recognized CD2, and six recognized gp42, a glycosylphosphatidylinositol (GPI)-anchored protein expressed by activated rat NK cells. In our initial studies of the remaining three mAbs (7D2, 2D1, and 6E2), we found that incubation of RNK-16 cells with any one of these prevented the subsequent binding of the others, suggesting that the three mAbs bind near the same epitope. To characterize the molecule recognized by these mAbs, RNK-16 cells were labeled with ^{125}I and then solubilized with NP-40. From the resulting lysate, 7D2 immunoprecipitated a broad band with a mean molecular mass of 35 kD under both reducing (Fig. 1 lane *A*) and nonreducing conditions (data not shown). Treatment of the immunoprecipitate with *N*-glycanase resulted in the appearance of a single, sharp band of 23 kD (Fig. 1 *B*). An identical result was obtained with 2D1 and with 6E2. Thus, these mAbs recognize a single chain glycoprotein of 35 kD that has a protein core of 23 kD.

As demonstrated using 7D2, the addition of these mAbs to [^{3}H]inositol-labeled RNK-16 cells stimulates a substantial (four- to fivefold) and sustained (>10 min) increase in inositol trisphosphate ($InsP_3$) and in its metabolites, inositol bisphosphate ($InsP_2$), and inositol phosphate ($InsP_1$) (Fig. 2 *A*), confirming that these mAbs activate the PI pathway in RNK-16 cells. Consistent with the link between PI turnover and Ca^{2+} fluxes, 7D2 elicits a sustained increase in the concentration of $[Ca^{2+}]_i$ in Indo-1-loaded RNK-16 cells (Fig. 2 *B*). This signaling response required the Fc region of mAb. $F(ab')_2$ fragments of mAb 7D2 failed to elicit an increase in $[Ca^{2+}]_i$, suggesting that interactions with Fc receptors are required for the agonist properties of the mAb (data not shown).

mAb 7D2 Stimulates Protein Tyrosine Phosphorylation. One mechanism by which cell surface receptors couple to the PI pathway is through the activation of protein tyrosine kinases. Accordingly, we determined whether the addition of 7D2 to RNK-16 leads to the appearance of tyrosine-phosphorylated proteins. We compared the response to 7D2 with the response to pairs of mAbs against CD2. CD2 is known to stimulate PI turnover in RNK-16 and, in human T cells, induces protein tyrosine phosphorylations (7, 12). As shown in Fig. 3, the tyrosine phosphorylation of several proteins is induced by 7D2, and at least two of these are also induced by stimulating CD2. In contrast, the addition of an isotype-matched mAb (OX-18) to rat MHC class I molecules does not lead to protein tyrosine phosphorylation. The combination of ionomycin and phorbol myristate acetate induces the tyrosine phosphorylation of only the 42-kD protein, suggesting that the majority of 7D2- and CD2-mediated tyrosine phosphorylations are not a consequence of PI turnover. Consistent with their inability to stimulate calcium signaling, $F(ab')_2$ fragments of mAb 7D2 failed to stimulate protein tyrosine phosphorylations (data not shown).

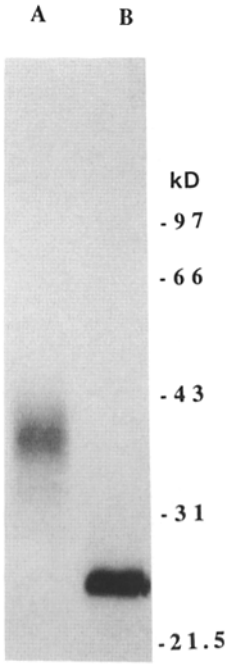

Figure 1. 7D2 immunoprecipitates a 30–40-kD glycoprotein from ^{125}I-labeled RNK-16 that has been solubilized in lysis buffer with 1% NP-40. Immunoprecipitates using 7D2 were either untreated (lane *A*) or digested with *N*-glycanase (lane *B*) before analysis under reducing conditions by SDS-PAGE and autoradiography.

mAb 7D2 Inhibits Killing by RNK-16 Cells. RNK-16 cells spontaneously lyse the NK-sensitive target, YAC-1 (7). As shown in Fig. 4, the killing of YAC-1 targets is inhibited by 7D2 but not by isotype-matched control mAbs to MHC class I molecules or to CD8 (both of which are expressed on RNK-16). The inhibitory effect of 7D2 is comparable with that of the CD2 mAb, OX-34, which has been shown to inhibit killing by RNK-16 (7). The ability of 7D2 to inhibit cytotoxicity establishes that 7D2 influences a cellular response of RNK-16 cells. It does not necessarily implicate the 7D2 determinant in mediating the responses of RNK-16 to YAC-1; by activating signaling pathways, 7D2 may desensitize RNK-16 to target-induced signals delivered through other cell surface molecules.

mAbs 7D2, 2D1, and 6E2 Recognize the OX-44 Molecule. 7D2, 2D1, and 6E2 stain virtually all mononuclear cells

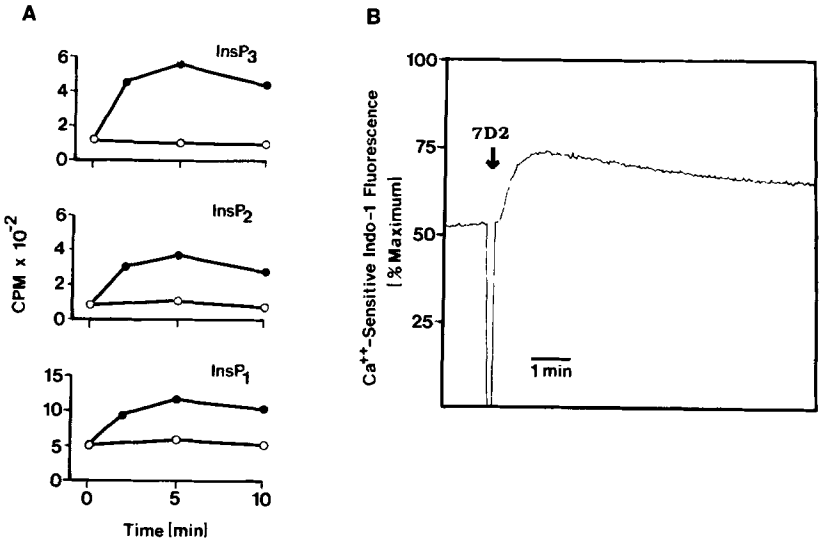

Figure 2. mAb 7D2 stimulates increases in InsPs (*A*) and $[Ca2+]_i$ (*B*) within RNK-16 cells. (*A*) [^{3}H]Inositol-labeled RNK-16 cells were incubated at 37°C in medium alone (*open circles*) or stimulated with 7D2 (*closed circles*; final concentration, 5 μg/ml). At the indicated time points cells were lysed in ice-cold 10% TCA. [^{3}H]$InsP_1$, [^{3}H]$InsP_2$, and [^{3}H]$InsP_3$ were extracted and resolved by anion exchange chromatography as described (7). The data represent the total of the indicated InsP in 5 × 10^6 cells at each time point and are representative of three separate experiments. (*B*) The Ca^{2+}-sensitive fluorescence of a suspension of Indo 1-loaded RNK-16 was monitored over time. $[Ca2+]_i$, calculated as described (7), was 236 nM in unstimulated cells. After the addition of 7D2 (final concentration, 5 μg/ml), $[Ca2+]_i$ rose to a maximal level of 589 nM and then fell to plateau of 390–400 nM. These data are representative of four separate experiments.

from rat spleen and lymph node as well as T cells and B cells purified from peripheral blood (not shown). In contrast to these results, the mAbs bound only a small minority (<10%) of thymocytes. The mass and tissue distribution of the molecule recognized by our mAbs, therefore, are similar to those reported for the MRC OX-44 molecule (1). Further, we observed that prior treatment of RNK-16 cells with OX-44 blocked the subsequent binding of 7D2, whereas a control mAb (OX-18) to MHC class I molecules did not (Fig. 5, *A–C*). In the converse experiment, prior treatment with 7D2 prevented the binding of OX-44 (Fig. 5, *D–F*). In additional studies, we found that OX-44 immunoprecipitated a molecule from RNK-16 cells that has the same electrophoretic mobility as the molecule immunoprecipitated by 7D2, and that preclearing lysates with 7D2 removed the molecule recognized by OX-44. Thus, we conclude that our mAbs and the OX-44 recognize the same cell surface molecule. The OX-44 mAb also stimulates a substantial increase in $[Ca^{2+}]_i$ in RNK-16, demonstrating that this mAb has agonist effects similar to the mAbs isolated by our screen (not shown).

mAb 7D2 Stimulates the Proliferation of Rat Splenic T Cells. To determine whether perturbation of the OX-44 molecule activates resting T lymphocytes, we studied nylon wool–nonadherent splenic mononuclear cells, a population that is composed predominantly of T cells but that also in-

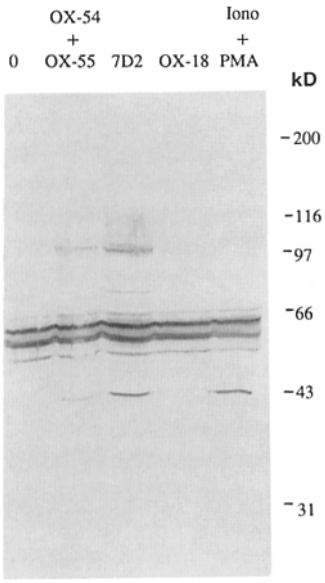

Figure 3. mAbs recognizing CD2 (OX-54 plus OX-55) or the OX-44 molecule (7D2) induce protein tyrosine phosphorylation in RNK-16 cells. RNK-16 cells were either incubated in medium alone (0), or with mAbs OX-54 plus OX-55, 7D2, OX-18 (anti-MHC class I), or with the combination of ionomycin (Iono; 1 μM) and PMA (40 ng/ml) for 2 min before lysis (all mAbs at 1 μg/10^6 cells). Cell lysates were analyzed under reducing conditions on a 9% polyacrylamide gel, transferred to PVDF paper, and blotted with an antiphosphotyrosine mAb. Each lane contains lysates from 2.5 × 10^6 cells.

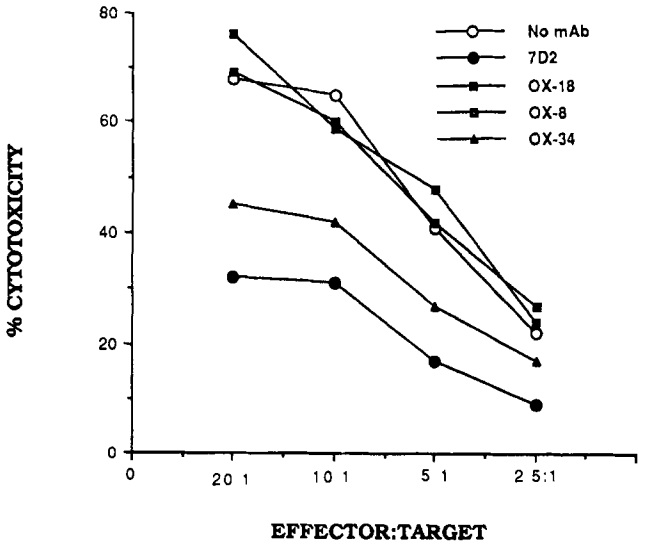

Figure 4. Natural killing by RNK-16 cells is inhibited by mAbs 7D2 and OX-34. Killing of YAC-1 target cells by RNK-16 is inhibited by the presence of either 7D2 or OX-34 (anti-CD2) but not by OX-18 (anti-MHC class I) or OX-8 (anti-CD8). RNK-16 were exposed to mAbs (1 μg/10^6 cells) just before the addition of target cells. Killing was assessed in an 18-h cytotoxicity assay. Each point is the mean of triplicate samples (SEM <10%). These data are representative of three separate experiments.

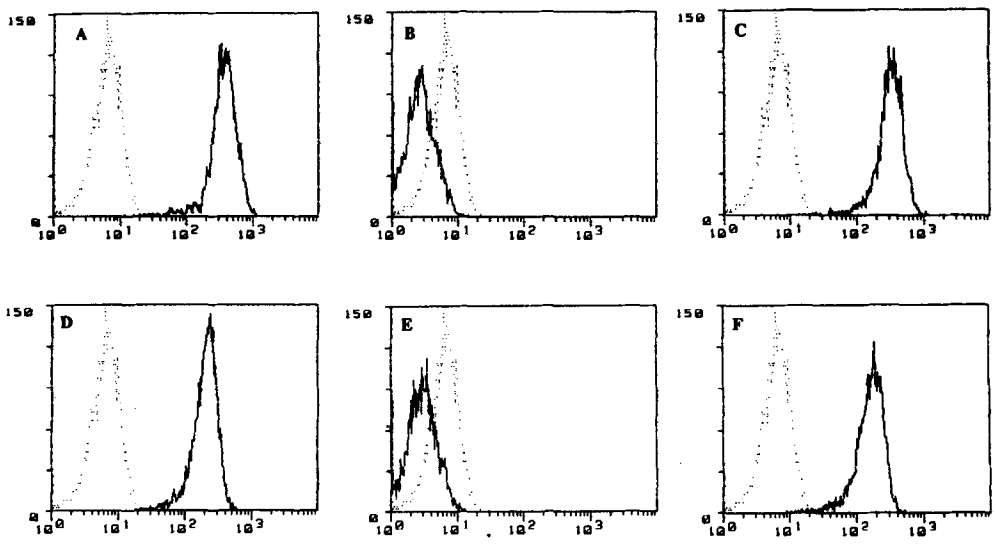

Figure 5. Reciprocal inhibition of binding to RNK-16 by mAbs 7D2 and OX-44. RNK-16 cells were either untreated (*A*), treated with unstained mAb OX-44 (*B*), or treated with unstained mAb OX-18 (*C*) on ice for 1 h before being stained with FITC-conjugated 7D2 and subjected to flow cytometric analysis. (*D–E*) RNK-16 cells were either untreated (*D*), treated with either unstained mAb 7D2 (*E*), or unstained mAb OX-18 on ice for 1 h before staining with FITC-conjugated OX-44 and analysis by flow cytometric analysis. The dotted line indicates fluorescence of a nonbinding FITC-conjugated IgG1 mAb (R73).

cludes accessory cells. The addition of 7D2 consistently stimulated proliferation, as assessed by the incorporation of [^{3}H]thymidine (Tables 1 and 2), whereas other mAbs to OX-44 molecule (OX-44, 2D1, and 6E2) did not (data not shown). The proliferative response of T cells to 7D2 was substantially less than that elicited by Con A but was somewhat greater than the response to optimal concentrations of mAb R73, which recognizes all TCR-α/β structures in the rat (Table 1) (13). The level of the proliferative responses to R73 in these experiments agreed with previously reported studies using this mAb (13). Both 7D2 and R73 are BALB/c IgG1 mAbs, indicating that the greater response to 7D2 is not a reflection of its isotype. Moreover, there were no kinetic differences between the responses to the two mAbs; peak proliferation to each mAb occurred at 48 h, and the response to 7D2 was greater than the response to anti-TCR at 24 and 72 h as well (not shown). In contrast to the results obtained with 7D2 and R73, there was no proliferative response to OX-18 (IgG1; anti-MHC class I), and little or no response to the CD2 mAbs OX-54 and OX-55 when these IgG1 mAbs were used individually (Table 1). As previously reported, the combination of OX-54 plus OX-55 was mitogenic and elicited levels of [^{3}H]thymidine incorporation similar to that of Con A (not shown) (14).

Table 1. *Proliferative Responses of Rat Splenic T Cells to 7D2*

	Stimuli*					
Exp. None	7D2	R73	OX-18	OX-54	OX-55	ConA
			cpm			
1 792‡	19,348	6,447	242	193	2,843	77,986
2 865	12,046	5,466	517	1,152	1,667	67,279
3 881	10,144	7,491	1,418	1,924	2,709	99,287

* Nylon wool–nonadherent splenic mononuclear cells (10^6 cells/ml) from Fischer rats were cultured for 48 h in microtiter plates. mAbs and Con A were added to a final concentration of 10 μg/ml. [^{3}H]Thymidine (1 μCi/well) was added 6 h before harvesting. Three independent experiments are shown.

‡ Mean of triplicate cell samples. SEM was <15% except for the OX-55 stimulation in Exp. 1 (SEM = 25%).

T cell proliferation in response to the combination of 7D2 and anti-TCR (R73) was greater than the sum of the individual responses (Table 2). There was no enhancing effect when either mAb was used in combination with OX-18 (not shown). Only a modest augmentation of the R73 response was elicited by the CD2 mAbs OX-55 (Table 1) or OX-54 (not shown). In view of the synergy observed here between 7D2 and R73, it is of interest that Beyers. et al. (2) had observed an enhanced increase in $[Ca^{2+}]_i$ when the OX-44 mAb and R73 were crosslinked on a rat T cell line.

The OX-44 Molecule Is Associated with CD2 on RNK-16 and Rat Splenic T Cells. Because mAb-induced perturbation of the OX-44 molecule transduces activation signals, and because of the relatively small size of its cytoplasmic domains, we addressed the possibility that the OX-44 molecule is noncovalently associated with other cell surface molecules. When RNK-16 cells were solubilized in a lysis buffer containing 10 mM CHAPS (but not 1% NP-40), a molecule of 50–60 kD coprecipitated with the OX-44 molecule (Fig. 6, lanes *1* and *3*). This coprecipitation was observed with three separate mAbs to the OX-44 molecule (2D1, 7D2, and OX-44) but not with a mAb to MHC class I molecules (OX-18; not shown). After Endo F treatment, the coprecipitating molecule migrated as a sharp band of 42 kD (Fig. 6, lane *2*). A band of this mass was usually observed after Endo F treatment of immunoprecipitates from NP-40 lysates (Fig. 6, lane *4*), but the intensity of the 42-kD band was variable and substantially less than that from the CHAPS lysate (compare Figs. 1 and 6). It appears, therefore, that small amounts of the 50–60-kD molecule are present in immunoprecipitates

Table 2. *Proliferative Responses of Rat Splenic T Cells to the Combination of 7D2 and a TCR mAb*

Exp.	None	Stimuli*					
		7D2	R73	7D2 + R73	7D2 + OX-55	R73 + OX-55	Con A
				cpm			
1	918‡	17,904	7,071	64,558	ND	ND	138,786
2	718	11,320	2,839	33,576	14,424	6,174	105,989
3	516	9,928	2,611	35,931	10,758	5,676	86,451
4	852	18,148	8,418	56,641	17,440	19,190	63,871

* Nylon wool–nonadherent spleen cells were stimulated as described in the legend to Table 1. The results of four independent experiments are shown.
‡ Mean of triplicate cell samples. SEM were <20%.

from NP-40 lysates and that deglycosylation substantially increases detection of this molecule.

In both glycosylated and deglycosylated forms, the coprecipitating molecule migrated on SDS-PAGE at the same mass as CD2 obtained by immunoprecipitation from RNK-16 lysates (not shown, but see below, Figs. 8 and 9). To determine whether the coprecipitating molecule was CD2, we excised the 42-kD band from the Endo F–treated immunoprecipitates of the OX-44 molecule and performed peptide maps. After digestion with either staphylococcal V8 protease (Fig. 7) or chymotrypsin (not shown), the peptide maps of the coprecipitating molecule were identical to those of Endo F–treated CD2 (Fig. 7). In contrast, a different map was generated by digestion of Endo F–treated MHC class I molecules, which migrated with a similar apparent mass (43 kD) on SDS-PAGE (Fig. 7).

To further confirm the identity of the 50–60-kD molecule as CD2, we took advantage of the differential abilities of CHAPS and NP-40 to permit coprecipitation. ^{125}I-surface-labeled RNK-16 cells were solubilized in lysis buffer containing CHAPS, which allows coprecipitation, and immunoprecipitations were performed using 2D1, a mAb to the OX-44 molecule. When the immunoprecipitates were washed in CHAPS-containing buffers, the 50–60-kD glycoprotein coprecipitated

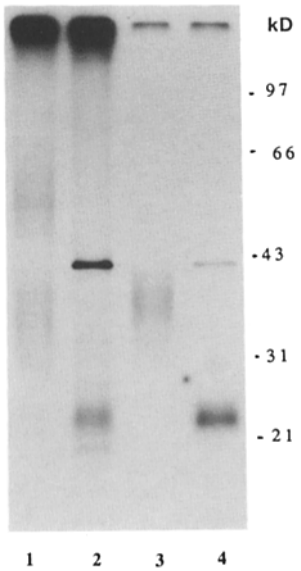

Figure 6. A 50–60-kD glycoprotein coprecipitates with the OX-44 molecule from RNK-16 cells. The OX-44 molecule was immunoprecipitated by mAb 2D1 from ^{125}I surface-labeled RNK-16 cells that had been solubilized in lysis buffer containing either 10 mM CHAPS (lanes *1* and *2*) or 1% NP-40 (lanes *3* and *4*). Immunoprecipitates were either untreated (lanes *1* and *3*) or digested with Endo F (lanes *2* and *4*) before analysis under reducing conditions by SDS-PAGE using an 11% polyacrylamide gel followed by autoradiography.

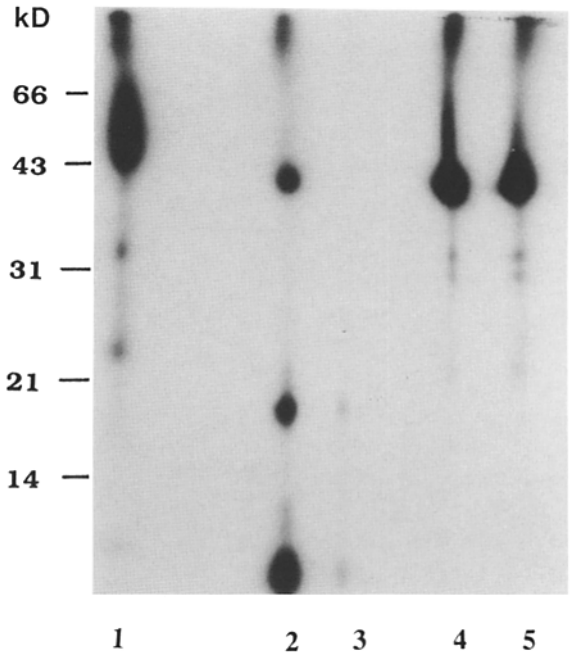

Figure 7. The peptide map of CD2 is identical to that of the protein that coprecipitates with the OX-44 molecule. Gel slices containing Endo F–treated CD2 (lanes *2* and *4*), Endo F–treated coprecipitating molecule (lanes *3* and *5*), and Endo F–treated MHC class I molecule (lane *1*) were partially digested with 1 μg (lanes *2* and *3*), 0.2 μg (lane *1*), or 0.05 μg (lanes *4* and *5*) Staphylococcal V8 protease and analyzed by SDS-PAGE and autoradiography. ^{125}I-labeled RNK-16 were solubilized in buffer containing 10 mM CHAPS, and immunoprecipitations were performed using mAbs OX-34 (anti-CD2), 2D1 (which recognizes the OX-44 molecule), and OX-18 (anti-MHC class I). The immunoprecipitates were digested with Endo F, resolved by SDS-PAGE, and visualized by autoradiography. The regions containing CD2, the 42-kD protein that coprecipitates with the OX-44 molecule, and MHC class I molecules were excised from the dried gels. These gel slices were rehydrated and treated with V8 protease according to the technique of Cleveland et al. (11). Samples were analyzed by SDS-PAGE (using 15% polyacrylamide gels) followed by autoradiography.

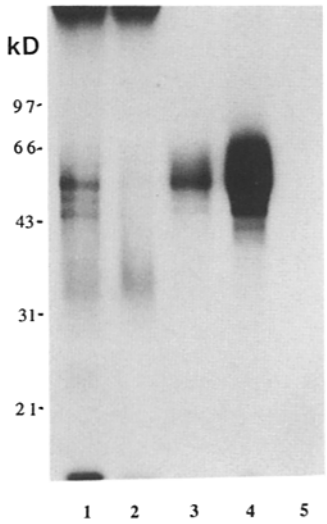

Figure 8. CD2 coprecipitates with the OX-44 molecule from CHAPS lysates of RNK-16. ^{125}I-labeled RNK-16 cells were solubilized in lysis buffer containing 10 mM CHAPS, divided into equal aliquots, and then subjected to immunoprecipitation using 2D1, a mAb to the OX-44 molecule. (Lane *1*) The immunoprecipitate was washed in 2 mM CHAPS and then eluted with Laemmli sample buffer. Alternatively, the immunoprecipitate was washed once in 2 mM CHAPS and then resuspended in buffer containing 1% NP-40. After a 30-min incubation, the immunoprecipitate was pelleted and eluted in Laemmli sample buffer (lane *2*). The NP-40 incubation buffer was then divided and subjected to immunoprecipitation using mAbs to CD2 (OX-34; lane *3*) or to CD8 (OX-8; lane *5*). (Lane *4*) CD2 was immunoprecipitated directly by mAb OX-34 from solubilized ^{125}I-labeled RNK-16. Samples were analyzed under reducing conditions on a 10% polyacrylamide gel, followed by autoradiography of the dried gel.

with the OX-44 molecule (Fig. 8, lane *1*). However, if the 2D1 immunoprecipitates were incubated in a buffer containing 1% NP-40 before SDS-PAGE, the 50–60-kD band dissociated from the immune complex (Fig. 8, lane *2*). The dissociated 50–60-kD molecule could then be immunoprecipitated from the NP-40 incubation buffer by mAb to CD2 (Fig. 8, lane *3*) but not to CD8 (Fig. 8, lane *5*), and it comigrated with CD2 that was immunoprecipitated directly from the RNK-16 lysate (Fig. 8, lane *4*). In contrast to these results, CD2 could not be detected in immunoprecipitates of MHC class I molecules (not shown).

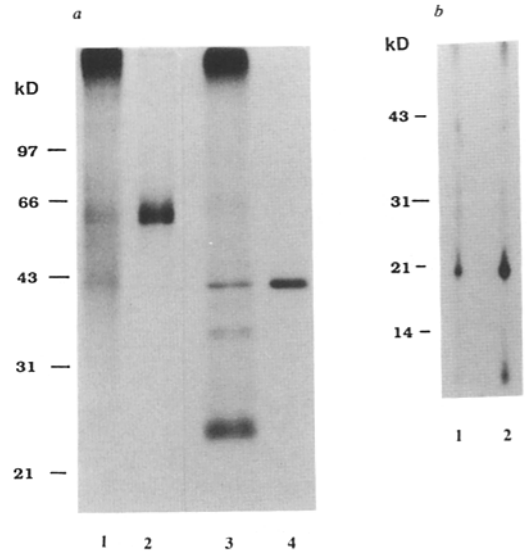

Figure 9. CD2 coprecipitates with the OX-44 molecule from rat splenic T cells. (*a*) CHAPS-containing lysates of ^{125}I-labeled T cells were subjected to immunoprecipitation using mAbs to the OX-44 molecule (2D1; lanes *1* and *3*) or CD2 (OX-34; lanes *2* and *4*). Samples were either untreated (lanes *1* and *2*) or digested with Endo-F (lanes *3* and *4*) before analysis by SDS-PAGE. (*b*) Peptide maps of the 42-kD deglycosylated protein in the 2D1 immunoprecipitate (lane *1*) and deglycosylated CD2 (lane *2*) were generated using staphylococcal V8 protease.

The OX-44 molecule obtained by immunoprecipitation from splenic T cells migrated at a higher apparent mass (35–45 kD) than the OX-44 molecule immunoprecipitated from RNK-16 cells. This difference in size was due to differential glycosylation; the deglycosylated molecules from RNK-16 and from T cells have the same apparent mass on SDS-PAGE. To determine whether CD2 coprecipitated with the OX-44 molecule from lysates of T lymphocytes, we surface labeled freshly isolated rat splenic T cells with ^{125}I and solubilized these cells in a CHAPS-containing buffer. From these lysates 2D1 immunoprecipitated a diffuse band of 35–50 kD (Fig. 9 *a*, lane *1*). Digestion with Endo F resulted in the appearance of the 23-kD deglycosylated OX-44 molecule. As in RNK-16 cells, it was associated with a 42-kD molecule that had the same electrophoretic mobility as deglycosylated CD2 (Fig. 9 *a*, lanes *3* and *4*). Peptide mapping studies confirmed that this coprecipitating 42-kD molecule was CD2 (Fig. 9 *b*). A third band of 31 kD also appeared in the Endo F digests of the 2D1 immunoprecipitates (Fig. 9 *a*, lane *3*), but this was not a consistent finding. The identity of this band is unknown.

Discussion

Our studies establish that the OX-44 molecule, when stimulated by mAbs, can transduce transmembrane signals. We also observe that CD2 coprecipitates with the OX-44 molecule, an indication that the two molecules are probably physically associated on the cell surface. Consistent with this possibility, we find that perturbation of the OX-44 molecule has effects on signal transduction and cellular responses that are similar to those elicited by stimulating CD2. In RNK-16 cells, each of the molecules can stimulate PI turnover, induce protein tyrosine phosphorylations, and inhibit target cell lysis. When stimulated by appropriate mAbs, either molecule can trigger the proliferation of T lymphocytes. We also find that stimulating the OX-44 molecule augments TCR-mediated proliferation. We did not observe a comparable enhancing effect of mAbs to rat CD2, but synergy between mAbs to CD2 and TCR has been reported in human systems (15). Taken together, these findings suggest that the OX-44 molecule may participate in CD2-mediated responses.

Our studies also indicate that the agonist properties of mAb 7D2 on RNK-16 cells depend upon the Fc region of the mAb and cannot be duplicated with $F(ab')_2$ fragments. This result suggests that signaling by the OX-44 molecule requires Fc receptor ligation, either as a costimulus or as a means of crosslinking the mAb. Recently, Spruyt et al. (16) have observed that Ca^{2+} signaling by CD2 mAbs in RNK-16 (but not T cells) also requires Fc receptor ligation.

The ability of mAb 7D2 to activate T cells in the absence of exogenous comitogens is shared by mAbs to a limited

number of molecules, most notably (but not exclusively) the TCR/CD3 complex and CD2 (14, 15). It should also be pointed out that our screen of nearly 200 hybridoma supernatants containing mAbs binding to RNK-16 led to the isolation of mAbs to only three cell surface structures: the OX-44 molecule, CD2, and gp42. The isolation of mAb to CD2 was anticipated, given the well-recognized ability of CD2 to signal in T cells and NK cells and our earlier finding that perturbation of CD2 by mAb generates a substantial InsP response in RNK-16 cells (7, 14, 17, 18). gp42 is a GPI-anchored protein that is selectively expressed by rat NK cells after activation by IL-2 (19, 20). The ability to stimulate PI turnover appears to be a general feature of GPI-anchored proteins on lymphocytes (21).

The gene encoding the OX-44 molecule has recently been cloned, and the predicted amino acid sequence indicates that it is the rat homologue of human CD53 (3–6). These molecules are members of a newly recognized family of glycoproteins that includes CD37, CD9, TAPA-1, ME491, CO-029, and Sm23 (3–6, 22–27). Members of this family lack a conventional leader sequence and are predicted to have four transmembrane domains with both their NH_2 and COOH termini located intracellularly (3–6, 22–27). When sequences for a particular family member are compared from different species, the putative cytoplasmic and transmembrane domains are highly conserved. For example, the NH_2-terminal hydrophilic region of the OX-44 molecule is identical to that of human CD53 (5, 6). Similarly, the cytoplasmic domains of human and rat CD37 are identical at 27 of 28 residues, an indication that these regions of CD37 are critical for its function (3, 4).

Although little is known concerning the physiological functions of this family of glycoproteins, recent studies of CD9, ME491, and TAPA-1 suggest that these molecules have roles in regulating signaling processes (25, 28–30). The addition of CD9 mAbs to platelets stimulates PI turnover, an increase in $[Ca^{2+}]_i$, granule secretion, and aggregation (28). Perturbation of CD9 also induces a physical association between CD9 and the platelet glycoprotein IIb-IIIa complex (the fibrinogen receptor), an event that could contribute to the conformational change necessary for exposure of the binding site for fibrinogen (29). ME491 was initially recognized on human melanoma cells (24). Kitani et al. (25) recently isolated a mAb (AD1) to the probable rat homologue of ME491 by screening mAbs for the ability to inhibit IgE-mediated release of histamine from a rat basophilic leukemia line. Although rat ME491 is not a known component of the high affinity IgE receptor (FcεRI), selected mAb to the FcεRI blocked the binding of AD1, suggesting that rat ME491 is in close physical proximity to the FcεRI (25). The ability of AD1 to inhibit IgE-mediated histamine release (but not the binding of IgE) suggests that rat ME491 may alter signaling by the FcεRI (25). mAbs to TAPA-1 were derived by screening for mAbs that inhibited the in vitro growth of lymphomas (23). These mAbs induce cell aggregation that is temperature sensitive and, therefore, may involve a TAPA-1-mediated signaling event (30). In certain cell lines, TAPA-1 has been shown to associate with a complex of cell surface molecules that includes Leu-13 (30). Thus, three molecules that are related to the OX-44 molecule have the capacity to modulate cellular responses and appear to physically associate with cell surface receptors.

Further studies are needed to determine whether the OX-44 molecule plays a role in the physiological activation of T cells and NK cells and to assess the functional importance of its interaction with CD2. CD2 has a large (116 amino acids) cytoplasmic domain, and this domain is required for CD2-mediated signaling (31, 32). CD2 does not signal when expressed in fibroblasts, suggesting that CD2 interacts with lineage-restricted molecules in order to transduce activation signals (31). It is highly probable that CD2 functionally interacts with several distinct cell surface structures. CD2-mediated signaling in human T cell lines is augmented by expression of the TCR/CD3 complex (33–35). A physical association between CD2 and this complex has been reported by some, but not other, investigators (36, 37). CD2 also appears to interact with the tyrosine phosphatase, CD45, which can be chemically crosslinked to CD2 on human T cells and which is required for signal transduction through CD2 and the TCR/CD3 complex (38–41). Like CD45, the OX-44 molecule is expressed by all mature leukocytes, suggesting that it is functional in cells that do not express CD2. It will be of interest to explore the signaling ability of the OX-44 molecule in these cells and to determine whether it can associate with ligand-binding molecules other than CD2.

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Address correspondence to John B. Imboden, Arthritis/Immunology Section (111R), Veterans Affairs Medical Center, University of California, San Francisco, 4150 Clement Street, San Francisco, CA 94121.

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