TAP, A NOVEL T CELL–ACTIVATING PROTEIN INVOLVED IN THE STIMULATION OF MHC-RESTRICTED T LYMPHOCYTES

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The expression of T lymphocyte effector functions requires cellular activation. Physiologically, these cells are stimulated upon interaction with specific antigen on an appropriate interacting second cell. There are multiple molecular interactions that contribute to T cell activation. First, the clonotypic T cell receptor interacts with antigen in association with MHC gene products on the surface of a target or accessory cell (1–3). This receptor is composed of α- and β-polypeptides with Ig-like structure and variability (4–8). In the human system, three nonvariable T3 proteins are intimately associated with the antigen (Ag)[1]/MHC receptor (9–11). It appears likely that murine T cells contain similar proteins; however, antibodies to such structures are currently unavailable (12, 13). Antibodies directed against the T cell receptor or T3 (in the human) can activate T cells (9, 14). Second, accessory molecules have been described. These include LFA-1 (15), L3T4 (16), and Lyt-2 (17); the latter two appear to correlate with the class of MHC molecules for which the T cell is specific (6, 18, 19). Antibodies against these structures do not activate, and it is currently thought that these molecules contribute to cellular interactions, possibly by increasing their avidity (6, 20). It appears that these molecules do not play an obligatory role in T cell activation (20, 21). Third, exogenous and/or endogenous cytokines may be required. For T inducer cells, monokines may be required in addition to T cell receptor triggering for production of IL-2 and/or IL-2-R (22–24). Interaction of IL-2 with its receptor, the latter of which requires activation for expression, drives the cell into cycle (25). Finally, other membrane molecules have been described on human T cells whose role is less well defined (e.g., T-11 [26] and 9.3 [27]). Murine homologues of these proteins have not been identified. Gunter et al. have observed that some mAbs against murine Thy-1 activate T cells (28).

During the course of studies analyzing the activation of murine inducer T cells, we have identified a novel T cell membrane antigen, T cell–activating protein (TAP). Antibodies directed against this molecule can directly activate T cells or influence their antigen-specific stimulation. The structure and expression of this protein is distinct from any of the above, previously described murine cell

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[1] *Abbreviations used in this paper:* Ag, antigen; RAMG, rabbit anti–mouse Ig; TAP, T cell activating protein; TAPa, TAP-associated protein.

TABLE I
Properties of mAbs

mAb	Specificity*	Isotype	Molecular mass of antigen‡	Functional effect§	Reference
			kD		
3A7	TAP	IgG2a	12	Activating	This report
1E7	TAP	IgG2b	12	Inhibitory	This report
1A8	TAP	IgG1	ND	Activating	This report
2D7	TAPa	IgM	16	None	This report
1H12	TAPx	IgG2a	ND	None	This report
M5/49‖	Thy-1	IgG2a	28	None	29
H013.4	Thy-1	IgM	28	None	30
11.4.1	H-2K^k	IgG2a	45/12	None	31
M1/42‖	H-2	IgG2a	45/12	None	32
MKD6	I-A^d	IgG2a	34/28	None	33
3JP	I-A^b	IgG2	34/28	None	34
10.2.16	I-A^k	IgG2b	34/28	None	31
HDP1¶	TNP	IgG1	—	None	¶

* 1H12 is referred to as anti-TAPx to indicate that its specificity has not been assigned to either the TAP or TAPa protein. 1A8 is tentatively assigned as anti-TAP on the basis of crossblocking, pattern of expression, and its functional effects.
‡ Reported as nonreduced for the TAP molecule. ND, not determined.
§ The anti-Ia mAbs inhibit T cell activation if they are directed against the Ia molecule that is corecognized by the T cell. For the experiments in this report they are used as isotype controls of irrelevant specificity and are therefore indicated as having no functional effect.
‖ Rat mAb.
¶ Drs. H. Urnovitz and R. Lynch, University of Iowa, unpublished data.

surface antigens. The characterization of the structure and function of this molecule is the subject of this report.

Materials and Methods

Animals. C57BL/10, B10.D2, B10.BR, SJL, C3H/Hej, AKR, A/J, $CXAF_1$, and CXBD mice ages 5–7 wk were purchased from The Jackson Laboratory, Bar Harbor, ME. BALB/c AnN were purchased from Charles River Breeding Laboratories, Inc., Wilmington, MA. B10.AQR mice were bred in our animal colony.

Animals used in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (Department of Health and Human Services, National Institutes of Health, publication 78-23, revised 1979).

Monoclonal Antibodies. The mAbs employed in this report are listed in Table I and/or below. The anti-TAP/TAPa mAbs were generated as follows: $CXAF_1$ mice were hyperimmunized with CXBD T cells and their sera were subsequently screened for functional effects on RF26.12, a B10 anti-I-E^d specific T cell hybridoma. Of 23 mice thus analyzed, 8 produced sera of varying titers that activated this T hybrid clone. A single mouse whose activating antibody titer was >1:1,600 was selected for fusion. 20×10^7 T cells were injected intravenously and intraperitoneally, and 3 d later splenocytes were harvested and fused with NSI myeloma cells by standard techniques (35). The resultant B cell hybridomas were screened for the ability of their supernatant to activate and/or bind to RF26.12. Positive wells were expanded and cloned by limiting dilution. The Ig isotypes were determined by Ouchterlony analysis with heavy chain–specific antisera. The remainder of the mAbs listed in Table I were kindly made available by the laboratories

that developed them as referenced, as were anti-L3T4 (GK1.5) (16), 14.4.4S (anti-Ia^7) (36), M5/114 (anti-Ia^d) (37), and anti-Lyt-2.2 (ADH.4) (38). mAb containing ascites or culture supernatant was prepared as previously described (39). Where indicated, antibody was purified from culture supernatant on a protein A–Sepharose column, or for IgM mAbs, by two saturated ammonium sulfate precipitations. The purified 3JP was provided by Dr. Man-Sun Sy, Harvard Medical School.

Immunofluorescence. Protein A–Sepharose–purified anti-TAP mAb and affinity-purified rabbit anti–mouse Ig (RAMG) were conjugated with FITC and 25 μl of 100 μg/ml was used per 10^6 cells. A 1:90 dilution of rat anti–mouse IgG2 mAb ascites (New England Nuclear, Boston, MA) and a 1:20 dilution of FITC goat anti–rat Ig, not crossreactive with mouse Ig (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) were also added at 25 μl per 10^6 cells. All incubations were performed at 0°C for 45 min followed by three washes. The precise sequence and combination of reagents are detailed in the individual experimental legends. For crossblocking experiments, the cell samples were preincubated with an unlabeled mAb for 45 min at 0°C, washed, then stained as indicated in the legend to Fig.1. After staining all samples were fixed with 1% paraformaldehyde and analyzed on a FACS II.

Immunoprecipitation. T cells were surface labeled with ^{125}I (1 mCi per 50×10^6 cells) with lactoperoxidase (40). For metabolic labeling, 150×10^6 cells were washed twice in PBS and resuspended in 40 ml of cysteine-free RMPI 1640 (M. A. Bioproducts, Walkersville, MD) containing 1 mCi [^{35}S]cysteine (New England Nuclear) and 10% dialyzed FCS and were incubated for 18 h at 37°C. Immunoprecipitations were performed as described by Borst (10, 11). Labeled cells were solubilized with 1% NP40 in 10 mM triethanolamine, pH 7.8, 150 mM NaCl, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml chymostatin, 1 μg/ml antipain, 1 μg/ml TLCK, and 10 mM iodoacetate, and incubated at least 45 min at 0°C. Labeled extracts were centrifuged at 15,000 *g* for 15 min followed by 100,000 *g* for 45 min. Labeled supernatants were precleared sequentially with fixed *Staphylococcus* Cowen strain I (The Enzyme Center, Boston, MA) for 18 h, three 45-min incubations with RAMG-normal mouse Ig preformed immune complexes, followed by 45 min with fixed *Staphylococcus*; all incubations were at 0°C. The precleared extracts were then incubated with preformed RAMG-mAb or control Ig complexes for 2 h at 0°C after which complex-bound material was pelleted and recentrifuged through a discontinuous sucrose-detergent gradient (10). The immunoprecipitated samples were analyzed by SDS-PAGE as described by Laemmli (41). Gels with ^{35}S-labeled samples were incubated with enlightening (New England Nuclear). Autoradiograms were exposed with an intensifying screen for 1–3 d at −70°C.

Cell Preparations and Cell Lines. Splenocytes were depleted of RBC by treatment with Tris-NH_4Cl. T cells were enriched by nylon wool fractionation (42). T cells were depleted by incubation with a cocktail of 240 μg H013.4 (anti-Thy-1.2), 1.5 ml GK1.5 supernatant (anti-L3T4), and 0.5 ml ADH4 supernatant (anti-Lyt-2.2) per 10^8 RBC free splenocytes for 30 min at 0°C, followed by 1 ml of 1:6 complement (Rabbit Low-Tox, Accurate Scientific Co., Hicksville, NY) for 30 min at 37°C. Ia-bearing cells were depleted by treatment with 1 ml M5/114 (anti-Ia) per 40×10^6 T cells followed by a 1:12 dilution of complement as described above. Con A blasts were prepared by culturing 5×10^6 nylon wool–purified T cells with 5 μg/ml Con A in media (see below) at 37°C for 48 h at which time they were adjusted to 10^6 cells/ml and both rat Con A supernatant (as a source of IL-2) and alpha methyl mannoside were added at a concentration of 5% and 20 mg/ml, respectively. After an additional 24 h incubation, T cell blasts were isolated on a Ficoll-Hypaque gradient. The following T cell hybrids are used in this report: RF26.12 (43) (B10 anti-$I\text{-}E^d$–specific), RF9.140 (44) (BALB/c anti-GAT + $I\text{-}A^d$–specific), RF26.98.47 (43) (B10 anti-$I\text{-}A^d$–specific), and RF7.24 (45) (BALB/c anti-GAT + $I\text{-}A^d$–specific). These hybrid clones are passaged in vitro in DME, supplemented as previously described (23), in the absence of other cell types. D10.G.4, an AKR anti-$I\text{-}A^k$ + conalbumin–specific, T cell inducer clone was kindly provided by Dr. C. Janeway, Jr., Yale University (14). The B10.A CH-1 (46) and BALB/c A202J (47) B lymphoblastoid cell lines were obtained from

Dr. Steve Herman (Dana Farber Cancer Institute, Boston, MA) and Dr. J. Kappler, (National Jewish Hospital, Denver, CO), respectively.

Cell Cultures and Assays. Microcultures with T cell hybridomas were prepared as previously described (44, 45). Briefly, 0.5–2 × 10^5 T cell hybrids were cultured with or without a source of accessory cells (1,660 rad x-irradiated splenocytes or A202J cells) in the presence or absence of mAb and/or antigen in duplicate or triplicate in a final volume of 200 μl of RPMI 1640 media supplemented as previously described (23). GAT was purchased from Vega Biotechnologies, Inc., Tucson, AZ. The precise constituents of hybrid and normal T cell cultures are detailed in the respective experimental protocols. After 18 h incubation at 37°C a 100-μl aliquot of supernatant was harvested, irradiated 8,000 rad, and assayed for IL-2 content with the HT2 indicator cell line (33, 45). Data are expressed as the mean cpm of label incorporated by 5 × 10^3 HT2 cells incubated in supernatants from duplicate or triplicate cultures as previously described (45). Microcultures with normal heterogeneous T cells were similarly constructed with 5 × 10^5 nylon wool–purified, unprimed T cells cultured with or without mAb in the presence or absence of 5 × 10^5 1,660 rad x-irradiated RBC free splenocytes or human monocyte IL-1, purified by hydroxy apatite adsorption, gel exclusion chromatography, and cation exchange HPLC (2.5–5 × 10^5 half-maximal U/mg; Collaborative Research, Waltham, MA). Cultures were incubated for 48 h at 37°C with 1 μCi of [^{3}H]thymidine added over the last 5 h of culture, unless otherwise indicated. Data are expressed as the mean cpm incorporated by triplicate cultures. The SEM for IL-2 and proliferative responses are usually <15%.

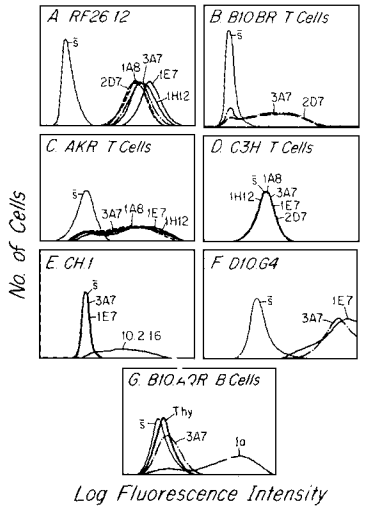

FIGURE 1. Expression of TAP determinants on lymphoid cells. The following cell populations were prepared for immunofluorescence analysis. (*A*) RF26.12 is a B10 anti-I-E^d–specific T cell hybridoma. (*B–D*) Nylon wool nonadherent, RBC-free splenocytes from the indicated strains. (*E*) CH-1 is a B lymphoblastoid cell line of B10.A origin. (*F*) D10.G4 is a T inducer clone of AKR origin. (*G*) B10.AQR RBC-free splenocytes treated with a cocktail of anti-Thy-1, anti-L3T4, and anti-Lyt-2 followed by complement. B10.AQR T cells bear TAP (data not shown). For staining, 10^6 cells were incubated for 45 min on ice sequentially with or without ($\bar{s}$) 100λ of the indicated mAb*-containing culture supernatant followed by FITC-RAMG (*A–D*) or monoclonal RAT anti–mouse IgG2 followed by FITC anti–RAT Ig (which is not crossreactive with mouse Ig) (*E–G*). The latter combination is employed to circumvent staining of Ig receptors on B cells. FITC-RAMG will stain B cells and demonstrates the absence of such cells from the T cell preparations. Stained samples were analyzed on a FACS II.

* 3A7, 1E7, and 1A8 = anti-TAP; 2D7 = anti-TAPa; 1H12 = anti-TAPx; 10.2.16 = anti-I-A^k, Thy = M5/49, Ia = 10.2.16 (anti-I-A^k) + 14.4.4.S (anti-Ia^7).

Results

Generation of Anti-TAP mAb. A variety of anti–T cell alloantisera were screened for the ability to modulate murine T lymphocyte function. To further characterize the relevant antibodies and their ligands, we derived B cell hybrids, which were screened for the production of mAbs that bound to, and/or caused activation of, a T cell hybrid. Five related mAbs were identified, whose features are summarized in Table I. The function and structure of the molecules detected by several of these mAbs have been analyzed and will be the subject of this report. These antigens have been provisionally termed T cell–activating protein (TAP) and TAP-associated protein (TAPa).

Expression of TAP Determinants on T Cells. The expression of TAP determinants on a variety of cell populations was tested by immunofluorescence and flow fluorocytometry. As illustrated in Fig. 1 *A*, all of the T cell hybrids that we have examined (>10) strongly express these antigens, as does the thymic lymphoma (BW5147) that was used in the construction of these hybrid clones (data not shown). The positive staining observed in this and subsequent experiments is specific as it is not observed with or without antibodies of irrelevant specificity followed by FITC anti-Ig reagents (Fig. 1 and data not shown). As shown in Fig. 1 *B*, ~70% of normal splenic T lymphocytes bear these antigens. Therefore, the expression of TAP proteins appears to define a major T cell subpopulation. In contrast to the expression of TAP determinants on a majority of peripheral T lymphocytes, only a small subpopulation of thymocytes, which are phenotypically mature, are TAP^+ (Yeh, E. T. H., H. Reiser, B. Benacerraf, and K. L. Rock, manuscript submitted for publication).

In the spleen, TAP antigens appear to be expressed only on T lymphocytes. This is illustrated in Fig. 1 *G*. In this experiment, T cell depleted spleen essentially fails to stain with anti-TAP mAbs. An anti-Ia mAb, which reacts with B lymphocytes and macrophages, stains the majority of cells in this population and serves as a positive control. The expression of TAP antigens on B lymphocytes was further analyzed with a B lymphoblastoid cell line of appropriate genetic background. As shown in Fig. 1 *E*, the CH-1 B cell line is not reactive with the anti-TAP mAbs, but stains brightly with anti-Ia mAbs. Under identical conditions, a normal (nonhybrid) T inducer clone stains intensely with the anti-TAP mAbs (Fig. 1 *F*). Taken together, these results suggest that resting B lymphocytes and non-B non-T cells either do not express TAP antigens or do so at significantly lower levels than T lymphocytes. However, B lymphocytes can express TAP determinants after activation (data not shown). Thus, the majority of B cell blasts stimulated with LPS for 96 h are stained with the anti-TAP mAbs, providing the lymphocytes are of the appropriate genetic background (Yeh, E. T. H., B. Benacerraf, and K. L. Rock, work in progress).

T cells from several inbred mouse strains are nonreactive with our anti-TAP mAbs. This is illustrated in Fig. 1 *D*. Lymphocytes from BALB/c, A/J, and C3H are not stained with any of the five antibodies (Fig. 1 *D*, and data not shown). In contrast, T cells from AKR, SJL, and all C57BL/6 background mice (C57BL/6, C57BL/10, and related MHC congenics) are positive (Fig. 1, *B–C* and data not shown). A more complete analysis of the genetics of TAP expression

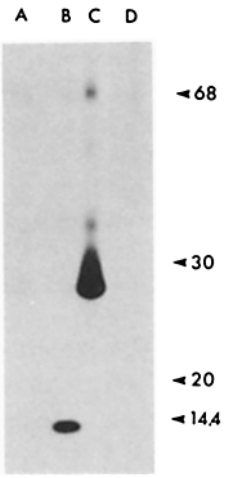

FIGURE 2. SDS Page analysis of ^{125}I-labeled TAP molecules. RF26.12 (B10 anti-I-E^{d}–specific T cell hybrid) was ^{125}I surface-labeled with lactoperoxidase. Cells were lysed with 1% NP40. Lysates were precleared with fixed staph A followed by three cycles with performed mouse Ig immune complexes followed by fixed staph A. Lysates were then immunoprecipitated with preformed complexes of: (*A*) mouse Ig (cIg); (*B*) 3A7; (*C*) Thy-1 (M5/49); (*D*) 2D7. Samples were eluted by boiling in SDS buffer and run on a 5–15% SDS-PAGE gel under nonreducing conditions.

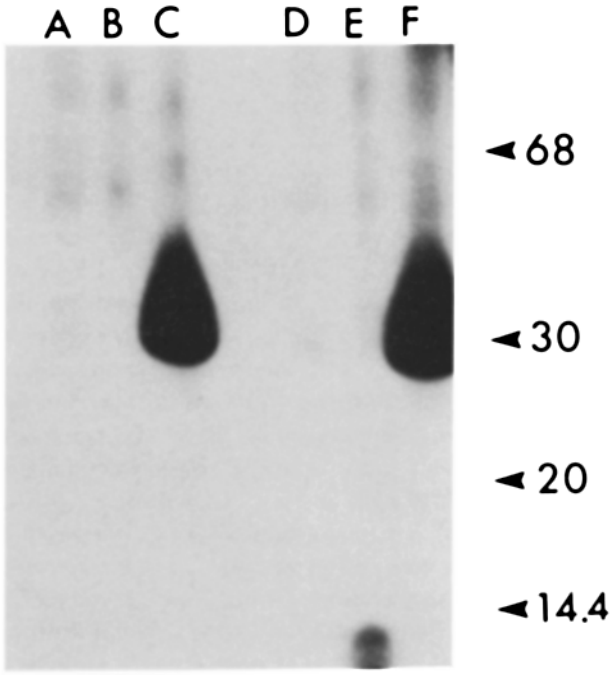

FIGURE 3. Immunoprecipitation of TAP molecules from Con A blasts. Con A blasts from BALB/ (*A–C*) or B10.D2 (*D–F*) T cells were ^{125}I surface-labeled with lactoperoxidase and handled as described in Fig. 2, except that immunoprecipitated samples were run on an 18% polyacrilamide gel. (*A, D*) cIg, (*B, E*) 3A7, (*C, F*) Thy-1.

is in progress and will be reported. The results with these negative strains again demonstrate the specificity of the immunofluorescence staining.

Biochemical Analysis of TAP Antigens. A T cell hybridoma expressing a high level of TAP antigen was chosen as a source of material for structural characterization. This clone was surface labeled with ^{125}I by lactoperoxidase and solubilized in NP-40. As shown in Fig. 2, the 3A7 anti-TAP mAb precipitates a major band that migrates with an apparent molecular mass of 12 kD on SDS-PAGE under nonreducing conditions. An identical band is observed after immunoprecipitation with the 1E7 anti-TAP mAb (data not shown). This band and those described below are specific as they are not observed in control precipitations with irrelevant antibody or anti-Thy-1.2 (Fig. 2). Similar results have been obtained in experiments with normal T cells. As shown in Fig. 3, 3A7 immunoprecipitates a 12-kD protein from ^{125}I surface-labeled Con A blasts. This molecule is not precipitated from BALB/c Con A blasts, which are genetically nonreactive with the anti-TAP mAbs. As shown, Thy-1 is precipitated from the BALB/c cells which serves as a positive control for labeling and precipitation. These results confirm the specificity of the immunoprecipitations with 3A7. To further characterize the TAP molecule and verify that the precipitated material is synthesized by the T cells, we metabolically labeled cells with ^{35}S cysteine. As shown in Fig. 4, the 3A7 and 1E7 mAbs specifically precipitate identically migrating 10–12-kD bands, sometimes resolved as a triplet. In contrast, the 2D7 mAb immunoprecipitates a distinct 16 kD band sometimes resolved as a doublet. This protein is not visualized by ^{125}I labeling (Fig. 2). The molecules identified by the mAbs 1A8 and 1H12 have not yet been characterized. A few additional minor bands have been observed in anti-TAP immunoprecipitations, whose significance requires further study. The above findings indicate that the mAbs detect at least two proteins that are distinguishable by size and differential labeling, which will be referred to as TAP (defined by 3A7, 1E7 mAbs) and TAPa (antigen detected by 2D7 mAb, which appears to be associated with TAP; see crossblocking below).

Crossblocking Studies. To define the relationship of TAP epitopes to one another, we have examined the effect of a saturating amount of one antibody (unlabeled) on the binding of a second, labeled mAb. As shown in Fig. 5, all five mAbs crossblock each other. All combinations of unlabeled vs. labeled anti-TAP/TAPa mAbs have been tested and have shown reciprocal inhibition (data not shown). A control antibody of irrelevant specificity (MKD6, anti-I-A^d, IgG2a)

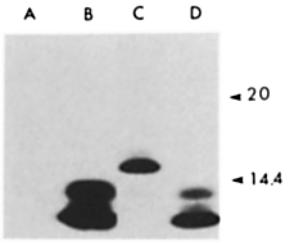

FIGURE 4. Metabolic label of TAP molecules. The RF26.12 hybrid was incubated with [^{35}S]cysteine (1 mCi/1.5 × 10^8 cells) for 18 h at 37°C and handled as described in Fig. 2 with the indicated mAb complexes. (*A*) cIg, (*B*) 3A7, (*C*) 2D7, (*D*) 1E7.

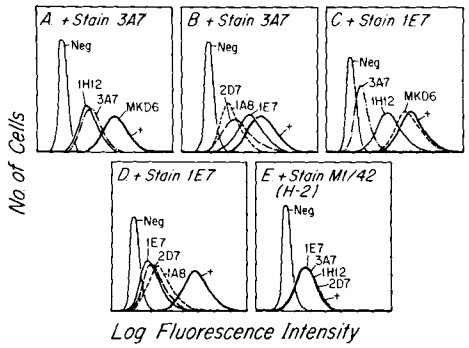

FIGURE 5. Effect of unlabeled anti-TAP mAb on the binding of other mAbs: crossblocking 10^6 T cell hybrids (RF26.12, B10 anti-IE^d–specific) were incubated in two stages. Stage 1: Inhibitor: incubated with (labeled curves) or without (+ curve) 5 μl of unconjugated mAb in the form of ascites at 0°C for 45 min, followed by 3 washes. Stage 2. Staining: incubated with FITC3A7 (*A* and *B*), FITC IE7 (*C* and *D*), or M1/42 (anti-H2) (*E*) for 0°C × 45 min, followed by FITC goat anti-rat mAb (*E*) which is noncrossreactive with mouse Ig. The fluorescence profile was analyzed with a FACS II. The profile without mAb in stage 1 is labeled "+" and the profiles with stage 1 mAb ascites are labeled with the source of the ascites: 3A7, 1E7, 1A8 = anti-TAP, 1H12 = anti-TAPx, 2D7 = anti-TAPa; MKD6 = IgG2 anti-IA^d (irrelevant specificity). Background staining with FITC anti-rat Ig is shown as the "Neg" curve. It is identical to hybrid without antibody or with an irrelevant FITC mAb (data not shown).

TABLE II

Activation of T Cell Hybrids by Anti-TAP mAbs

Exp.	Hybrid Specificity	mAb*	Specificity	APC	H-2	Antigen 100 μg/ml	cpm
1	RF26.12 (B10	—		—	—	—	1,689
	anti-I-E^d)	3A7	TAP	—	—	—	89,710
		1A8	TAP	—	—	—	10,821
		1E7	TAP	—	—	—	1,014
		2D7	TAPa	—	—	—	1,194
		1H12	TAPx	—	—	—	1,190
		3JP	I-A^b	—	—	—	736
		—		A20	H-2	—	62,568
2	RF9.140 (B/c	—		—		—	848
	anti-I-A^d + GAT)	3A7	TAP	—		—	93,748
		3JP	I-A^b	—		—	693
		—		A20	H-2^d	—	707
		—		A20	H-2^d	GAT	99,927
3	RF26.89.47 (B10	—	TAP	—		—	945
	anti-I-A^d)	3A7	TAP	—		—	54,933
		11.4.1	H-2K^k	—		—	874
		—		BALB/c	H-2^d	—	22,143

Microcultures were prepared with 1–2 × 10^5 T cell hybrids with or without the indicated mAb, in the presence or absence of a source of APC (experiments 1 and 2, 10^5 Ia$^+$ A20 cells; experiment 3, 10^5 irradiated splenocytes) and antigen where indicated, in a final volume of 200 μl. Cultures were incubated at 37°C for 18 h at which time a 100-μl aliquot of supernatant was removed x-irradiated and assayed for IL-2 content.

* Exps. 1–2, 1:50 dilution of dialyzed ascites; exp. 3, 1:4 dilution of 3A7 culture supernatant and 10 μg/ml of purified 11.4.1 antibody.

fails to block, and the anti-TAP mAbs fail to inhibit the binding of an anti-H-2 (Fig. 5), anti-L3T4, or anti-T cell receptor antibody (data not shown). These results suggest a close proximity or conformational dependence of the TAP/TAPa epitopes. Given this apparent relationship on the cell surface and the similar pattern of expression, we have referred to the distinct protein detected by 2D7 as TAP associated (TAPa).

Function of TAP Molecules: T Cell Activation by Anti-TAP mAbs. The impetus for the present study was the initial observation that an anti-T cell alloantiserum caused T cell activation. As shown in Table II, two of the anti-TAP mAbs (3A7 and 1A8) activate T cell hybridomas to produce IL-2. The 3A7 mAb is more active than 1A8, and induces maximal activation (equivalent to maximal antigen or lectin responses). Purified 3A7 mAb is sufficient for stimulation and can be active at nanogram amounts (e.g., half-maximal activation at 10 ng/ml), although individual hybrids may differ in the amount of antibody required for activation (data not shown). This effect is highly specific since mAbs of the same isotype but of unrelated specificity and/or that bind other T cell determinants (e.g., anti-H-2, anti-Lyt-1, anti-L3T4, or anti-Thy-1.2), fail to stimulate the hybrids (Table II and data not shown). All T cell hybrids that have been examined (>10) are activated, irrespective of the T cells clonotypic specificity (Table II and data not shown). The TAP-positive BW5147 tumor does not produce IL-2 upon stimulation with anti-TAP mAbs (data not shown). This is not surprising as it fails to produce IL-2 even upon mitogen stimulation (reference 48 and data not shown). However, the TAP antigens expressed by the BW5147 tumor are functional when introduced into competent cells, since antigen-specific BALB/c (genetically TAP^-) × BW5147 T inducer hybrids are activated by 3A7 (Table II).

TABLE III

Activation of Normal Heterogeneous T Cells by Anti-TAP mAbs

Exp.	T cell Strain (TAP) (5×10^5)	mAb	Specificity	[^{3}H]Thymidine incorporation (cpm)
1	B10.D2 (+)	—		3,517
		3A7	TAP	83,644
		1A8	TAP	13,549
		2D7	TAPa	2,085
2	B10.D2 (+)	—		3,262
		3A7	TAP	19,185
		1E7	TAP	2,770
		1H12	TAPx	3,462
	BALB (−)	—		2,265
		3A7	TAP	1,161

Microcultures were prepared with 5×10^5 nylon wool–passed lymph node from the indicated strains, in the presence or absence of mAb as 1:200 dilution of ascites (anti-TAP). In Exp. 2, 5×10^5 1,660-rad irradiated RBC and free syngeneic splenocytes were added as a source of accessory cells. Cultures were incubated at 37°C for 48 h, and incorporation of [^{3}H]thymidine was measured over the final 6–8 h. As a control for the competence of the BALB/c T cells in Exp. 2, they were tested in an MLR by culturing with and without 5×10^5 syngeneic or allogenic irradiated splenocytes for 96 h. [^{3}H]Thymidine incorporation (cpm): no APC, 1,906; syngeneic APC, 6,310; allogeneic APC, 73,541.

TABLE IV

Accessory Cell/IL-1 Dependence of Anti-TAP Activation of Normal T Cells

Splenocyte strain (5×10^5) (TAP)	Treatment*	mAb	[^{3}H]Thymidine incorporation in cells cultured with:		
			Alone	APC	IL-1
B10.D2 (+)	—	—	1,925	—	—
	—	3A7	9,227	—	—
	NW anti-Ia + C′	—	1,998	1,644	3,461
	NW anti-Ia + C′	3A7	2,879	19,780	33,232

Microcultures were prepared with spleen cells treated as indicated in the presence or absence of mAb as 1:100 dilution of ascites (anti-TAP). Where indicated, 5×10^5 1,660 rad irradiated RBC and free syngeneic splenocytes were added as a source of accessory cells. Where indicated, 5 U/ml purified IL-1 were added. Cultures were incubated at 37°C for 48 h and incorporation of [^{3}H]thymidine measured over the final 6–8 h.

* NW, nylon wool passage, anti-Ia + C′ with M5/114.

As shown in Table II, the other three anti-TAP/TAPa mAbs fail to activate T cell hybridomas. These mAbs have been tested over a wide range of concentration with identical results. Further, preliminary experiments have not revealed stimulatory activity when these nonactivating mAbs are immobilized on Sepharose beads to increase avidity and crosslinking (data not shown). Similarly, the presence of accessory cells in culture does not alter their behavior (data not shown).

We next investigated the effect of the anti-TAP mAbs on normal, heterogeneous T cells. As shown in Tables III and IV, the anti-TAP mAbs are mitogenic for normal T cells. Several points should be noted. First, the pattern of activity is qualitatively identical to that observed with the T cell hybridomas. Of the five anti-TAP/TAPa mAbs, only 3A7 and 1A8 are stimulatory, with the former inducing a stronger response (Tables III and IV). The degree of proliferation is significantly less than observed with an optimal Con A response (data not shown). Second, the stimulation is specific; control mAbs, including those that bind the T cell surface (e.g., anti-H-2 and anti-Thy-1.2) do not cause activation (Tables III and IV, and data not shown). Also, T cells genetically nonreactive with the anti-TAP mAbs are not activated (Table III). Third, the assay for activation is proliferation, which is measured at 48 h of culture. Therefore, the antibodies are capable of rapidly driving at least some T cells into cell cycle. Fourth, the activation of normal T cells may be more complex than for the T cell hybridomas. Thus when T cells were rigorously depleted of accessory cells by anti-Ia + complement treatment, they failed to respond to 3A7 stimulation (Table IV). This response is restored by the addition of irradiated accessory cells or highly purified IL-1 (Table IV). Taken together these results clearly generalize the phenomenon of anti-TAP T cell stimulation.

Effect of Anti-TAP mAb on Antigen-specific T Cell Responses. To address whether the TAP/TAPa molecules might be involved in physiologic (antigen-specific) T cell stimulation, we tested the effect of the mAbs on specific T cell responses. We first asked what effect, if any, the activating anti-TAP mAbs have on such responses. As shown in Fig. 6, a suboptimal amount of the activating anti-mAbs

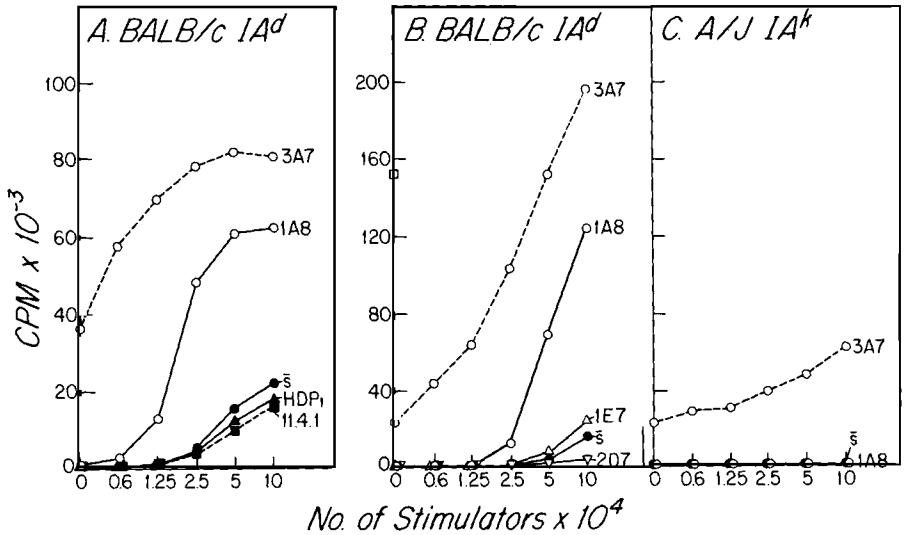

FIGURE 6. Effect of activating anti-TFA mAbs on antigen-specific stimulation of T cell hybridomas. Microcultures were prepared 10^5 RF26.98.47* with or without the indicated number of irradiated BALB/c (H-2^d) or A/J (H-2^a) splenocytes (as a source of allogeneic stimulators) in the presence or absence of the indicated mAbs (see below). Cultures were incubated at 37°C for 18 h at which time an aliquot of supernatant was removed and assayed for IL-2 content. *B* and *C* are from the same experiment.

* RF26.98.47 is an alloreactive, I-A^d specific hybridoma, and it does not react with I-A^k (A/J = I-A^k). 3A7, 1:16 (--○--) or 1:4 (□) dilution of culture supernatant. 1A8, 1:4 dilution of culture supernatant (—○—). For this preparation, the amount of antibody is not sufficient to directly activate. 1E7, 1:4 dilution of culture supernatant (△). 2D7, 1:4 dilution of culture supernatant (▽). 11.4.1, IgG2a, a (anti-H2K^k), 10 μg/ml purified mAb (■). HDP_1, IgG1, (anti-TNP mAb), 10 μg/ml purified mAb (▲).

TABLE V

Augmenting Effect of Activating Anti-TAP mAb on an Antigen-specific T Cell Hybrid

Hybrid (specificity)	B/C APC	GAT	mAb	Specificity	cpm
		μg/ml			
RF 9.140 (GAT + I-A^d)	+	—		—	466
	+	—	1A8	TAP	730
	+	—	M5/49	Thy-1	926
	+	12.5		—	2,842
	+	12.5	1A8	TAP	10,999
	+	12.5	M5/49	Thy-1	2,228
	+	25		—	4,840
	+	25	1A8	TAP	20,472
	+	25	M5/49	Thy-1	7,043

Microcultures were prepared with 5×10^4 T cell hybrids and 10^6 irradiated BALB/c splenocytes (as a source of antigen-presenting cells), with or without the indicated limiting amount of antigen, in the absence or presence of a 1:4 dilution of the indicated mAb-containing supernatants. For this preparation, the concentration of 1A8 mAb is limiting and insufficient to cause direct activation. Cultures were incubated for 18 h at 37°C at which time an aliquot of supernatant was removed and assayed for IL-2 content.

synergise with stimulation through the T cell antigen/MHC receptor. Thus, as illustrated, 1A8 and 3A7 both decrease the threshold for antigenic stimulation and markedly increase responses of the I-A^d-specific, alloreactive hybrid, RF26.98.47. This effect requires receptor stimulation since it is not observed with stimulators bearing the inappropriate Ia molecules (A/J, H-2^a). In this experiment a weak enhancement of the 3A7 response (but not 1A8) is observed with higher numbers of A/J cells (Fig. 6*C*). This latter finding may reflect a presentational influence; however, its magnitude is modest and not sufficient to account for the dramatic effect observed with the appropriate antigen-bearing stimulator. This is further demonstrated with antigen-specific hybrids since, as shown in Table V, both specific antigen and accessory cells are required for the antibody effect. In both of these experiments, the antibody effect is on the T cell since the BALB/c and A/J presenting cells are genetically nonreactive with the anti-TAP mAbs. As shown, several control mAbs fail to affect responses (Fig. 6, Table V). It should be noted that at similar limiting concentrations, the 1E7 and 2D7 mAbs fail to augment responses. Similar results have been observed with normal heterogeneous T cell responses (data not shown). These results indicate that the activating anti-TAP mAbs are capable of positively modulating antigen-specific T cell responses.

We next examined the effect of the nonactivating mAbs on antigen-specific hybridoma responses. As just noted, at limiting concentration these antibodies neither enhance nor inhibit responses. However, at high concentration the 1E7 anti-TAP mAb is moderately inhibitory (Table VI). This effect is specific as inhibition is not observed with control antibodies, including several that bind other T cell membrane proteins. In this experiment, the locus of inhibition is the T cell since the accessory cells in culture are genetically unreactive with this antibody. In contrast to this finding, the nonactivating mAbs 2D7 and 1H12 fail to affect responses even at high concentration. Therefore, antibody binding to the TAPa protein (defined by 2D7) does not interfere with its function, if indeed it has one. The observed activating, augmenting, and inhibitory effects of the

TABLE VI

Anti-TAP Inhibition of Antigen-specific T Cell Activation

Hybrid (specificity)	B/c APC	GAT	mAb	Specificity	cpm
RF 7.24 (GAT + I-A^d)	+	—	—	—	731
	+	+	—	—	49,852
	+	+	1E7	TAP	7,094
	+	+	1H12	TAPx	40,760
	+	+	2D7	TAPa	32,180
	+	+	H013.4	Thy-1	38,166
	+	+	3JP	I-A^b	38,517
	+	+	M5/49	Thy-1	45,255

Microcultures were prepared with 5×10^4 T cell hybrids, 5×10^5 irradiated BALB/c splenocytes (as a source of antigen-presenting cells) with or without 100 μg/ml of GAT in the presence or absence of 100 μg/ml of purified mAb (except M5/49, which was added as a 1:4 dilution of culture supernatant). Cultures were incubated for 18 h at 37°C after which time supernatant was removed and assayed for IL-2 content.

anti-TAP mAbs suggest that TAP participates in physiologic antigen-specific T cell stimulation.

Discussion

The present study was initiated to investigate cell surface proteins involved in murine T cell immune function. In this report we describe the characterization of a novel membrane T cell–activating protein (TAP). The TAP antigen is expressed on ~70% of normal peripheral T cells and has an allelic polymorphism in this expression. All T cell hybridomas examined bear TAP as does a T inducer cell clone. Preliminary experiments suggest that this molecule is present on all T inducer cells (Yeh, E. T. H., and K. L. Rock, work in progress). It will be of interest to define the characteristics of the 30% of normal T cells that either lack or express low levels of TAP.

The structure of the TAP molecule has been partially characterized. When immunoprecipitated from ^{125}I surface-labeled T cells it migrates as a 12-kD band on SDS-PAGE under nonreducing conditions. Preliminary experiments show an increase in molecular mass to ~17–19 kD upon reduction (data not shown). This may reflect intrachain disulfide bonds. Metabolic labeling experiments have confirmed that TAP is a protein synthesized by the T cells. Two slightly smaller bands are also observed by internal label, which may represent precursors.

In parallel internal label experiments, the 2D7 mAb, which was derived from the same fusion as the anti-TAP mAbs, precipitates distinct ~16-kD bands. This molecule is not labeled by lactoperoxidase catalyzed iodination and its mobility is not altered upon reduction (data not shown). By these criteria, this protein is distinct from TAP. Nevertheless, the 2D7 mAb efficiently crossblocks the binding of anti-TAP mAbs to the cell surface and vice versa. The most straightforward interpretation of this finding is that the protein detected by the 2D7 mAb is closely associated with TAP molecules on the cell surface. This point requires further study. However, on the basis of these observations, the 16-kD protein has provisionally been labeled TAPa.

What is the relationship of the TAP molecule to previously described T cell antigens? It is clearly distinct from the most well-characterized murine differentiation antigens. Thus, on the basis of cellular expression, allelism, and molecular weight, it is distinct from Thy-1, Lyt-1, Lyt-2, L3T4, LFA-1, and the T cell receptor (4, 5, 15, 16, 49–51). The immunization from which the TAP mAb was produced should contain antibodies against Ly-6 (52). Presently, the Ly-6 locus appears to encode several hematopoietic antigens (53–55). However, the pattern of cellular expression and the molecular structure of TAP makes it appear distinct from previously described Ly-6-related molecules (53–57). Studies are underway to further analyze the genetics of TAP expression.

Several of the features of TAP are reminiscent of the human T cell receptor–associated T3 (CD3) molecules (δ, γ, and ϵ), including antibody mediated activation and inhibition (6, 9–12). Antibodies directed against murine T3 molecules have not been described. However, it appears that murine T cells have homologous structures since they contain a transcriptionally active T3 δ gene and T cell receptor–associated proteins have been observed under certain conditions (12, 13). Several observations suggest that TAP is not the murine homologue of the

previously described T3 proteins. Its molecular weight is significantly smaller than either the human proteins or the predicted/observed proteins in the mouse (12, 13). Furthermore, its cell distribution appears distinct as it is not a pan–T cell antigen and may be expressed on activated B cells. Thus, the available evidence suggests that TAP is not a T3 homolog, although it is possible that it is associated with such structures. It should be noted that by similar criteria TAP is distinct from other described human T cell antigens, including proteins with which antibody-induced activation has been observed (e.g., T11 [58] and 9.3 [27]). We conclude that TAP appears to be a novel T cell membrane antigen.

The TAP molecule is of particular interest because antibody binding has profound functional effects, the most prominant of which is cellular activation. In this context, two points should be noted: first, soluble anti-TAP mAb is sufficient for activation. Although many antireceptor antibodies require immobilization to be stimulatory, there is clearly precedence for both soluble anti–B and anti–T cell receptor antibodies causing activation (14, 59). In our system, the nonactivating anti-TAP mAb (e.g., 1E7) does not become stimulatory upon immobilization (preliminary experiments, data not shown). Second, activation of most T cell hybrids appears to require only receptor triggering without other second signals (e.g. IL-1). Thus, most T hybrid clones respond directly to lectins, anticlonotypic mAbs, and will function with fixed antigen-presenting cells, provided antigen processing has occurred (60, 61). Therefore, a direct activating effect of soluble antibody is not surprising, and it clearly demonstrates the direct triggering potential of this molecule.

Normal T cells are also stimulated by anti-TAP mAbs. Addition of 3A7 or 1A8 anti-TAP mAbs to cultures of heterogeneous T cells results in a modest, but significant proliferative response. This observation generalizes the activating property of the TAP molecule. It also demonstrates that anti-TAP mAb stimulation of T cells is sufficient to drive T cells into cell cycle. This response of normal T cells, however, requires additional signals provided by either accessory cells or purified IL-1. It appears, therefore, that the conditions for anti-TAP stimulation are similar to those required for antigen/Ia or lectin stimulation of normal T cells (14, 25, 62, 63).

Most anti-T cell antibodies are not stimulatory. The ability of anti-TAP mAbs to activate T cells indirectly implies a receptor/triggering role for the TAP molecule. Indeed, most well-characterized examples of such effects involve antibodies directed against the physiologic triggering structures (9, 14, 61). Therefore, it was of interest to determine whether TAP participates in specific immune stimulation. When anti-TAP mAbs are added to cultures of antigen-stimulated T cells, two distinct effects are observed, depending on the mAb. First, the activating anti-TAP mAbs (3A7 and 1A8) synergise with antigen-specific stimulation. This synergy could arise if anti-TAP-TAP is augmenting the function of the T cell receptor or if it is generating a signal that acts in concert with the latter. Our data do not distinguish between these possibilities. The nonactivating anti-TAP and anti-TAPa mAbs do not augment responses. Second, a nonactivating anti-TAP mAb (1E7) interferes with antigen stimulation of T cell hybrids. Despite having this opposite functional effect, the 1E7 mAb appears to react with the same protein as the 3A7 mAb. There is precedence for

different mAbs against the same molecule having distinct functional effects (58, 64, 65). Our data does not distinguish whether this difference is attributable to a property of the mAb (e.g., affinity or isotype) or the determinants that are recognized (e.g., reacting with distinct functional domains on the TAP molecule). As noted, the 3A7 and 1E7 mAbs crossblock one another's binding. Taken together, these results suggest that the TAP molecule participates in immune stimulation. It is possible that TAP participates in the T cell receptor complex or alternatively it might be involved in a distinct molecular triggering event. Our data do not distinguish between these or other possibilities. Nevertheless, these findings suggest that the membrane molecules involved in T cell activation may be more complex than previously described. In this context, two points are intriguing. First, TAP could represent the first example of a murine T cell subset–specific activating protein since it is not detectable on all T cells. Second, the TAP molecule is expressed on phenotypically and functionally mature thymocytes but not cortical (double positive) cells, the latter of which can express the T cell receptor but are not responsive (Yeh, E. T. H., H. Reiser, B. Benacerraf, and K. L. Rock, manuscript submitted for publication). This raises the possibility that TAP expression is involved in the acquisition of immunocompetence. Future studies will analyze these points as well as the molecular basis for TAPs' functional properties.

Summary

Five mAbs have been generated and used to characterize TAP (T cell activating protein) a novel, functional murine T cell membrane antigen. The TAP molecule is a 12-kD protein that is synthesized by T cells. By antibody crossblocking, it appears to be closely associated with a 16-kD protein on the T cell membrane also identified with a novel mAb. These molecules are clearly distinct from the major well-characterized murine T cell antigens previously described. Antibody binding to TAP can result in the activation of MHC-restricted, antigen-specific inducer T cell hybridomas that is equivalent in magnitude to maximal antigen or lectin stimulation. This is a direct effect of soluble antibody and does not require accessory cells or other factors. The activating anti-TAP mAbs are also mitogenic for normal heterogeneous T lymphocytes in the presence of accessory cells or IL-1. In addition, these antibodies are observed to modulate specific immune stimulation. Thus, the activating anti-TAP mAbs synergise with antigen-specific stimulation of T cells, while a nonactivating anti-TAP mAb inhibits antigen driven activation. These observations suggest that the TAP molecule may participate in physiologic T cell activation. The possible relationship of TAP to known physiologic triggering structures, the T3-T cell receptor complex, is considered. TAP is expressed on 70% of peripheral T cells and therefore defines a major T cell subset, making it perhaps the first example of a murine subset-specific activating protein.

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Note Added in Proof: The surface ^{125}I-labeled TAP antigen has recently been resolved as a doublet on SDS-PAGE. Preliminary studies with the 1A8 and 1H12 mAbs demonstrate that they precipitate the 12 kD TAP protein.

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