The Monomeric Guanosine Triphosphatase rab4 Controls an Essential Step on the Pathway of Receptor-mediated Antigen Processing in B Cells

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Summary

Each member of the rab guanosine triphosphatase protein family assists in the regulation of a specific step within the biosynthetic or endocytic pathways. We have found that the early endosome-associated rab4 protein controls a step critical for receptor-mediated antigen processing in a murine A20 B cell line. Expression of the dominant negative rab4N[121]I mutant dramatically inhibited the processing and presentation of ovalbumin, λ cI repressor, or rabbit immunoglobulin G internalized as antigens by B cell antigen receptors or transfected Fc receptors. This defect did not reflect a block in antigen endocytosis or degradation, and transfected cells remained completely capable of presenting exogenously added ovalbumin and λ repressor peptides. Most remarkably, rab4N[121]I-expressing cells were undiminished in their ability to present each of these antigens when whole proteins were internalized at high concentration by fluid-phase endocytosis. Thus, expression of the rab4N[121]I selectively inactivated a portion of the endocytic pathway required for the processing of receptor-bound, but not nonspecifically internalized, antigens. These results suggest that elements of the early endosome-recycling pathway play an important and selective role in physiologically relevant forms of antigen processing in B cells.

Key words: endocytosis • endosomes • B cell • major histocompatibility complex class II • recycling

Presentation of immunogenic peptides bound to MHC class II molecules to CD4^{+} T cells typically involves the endocytosis of exogenous Ag. Internalized Ags are proteolyzed and the resulting peptides are loaded onto MHC class II α/β dimers, either newly synthesized or internalized from the plasma membrane (1–3). Although these events can occur in most cell types, professional APCs have a number of specializations that contribute to their Ag processing efficiency. For example, B cells express Ag receptors that bind and internalize only that Ag for which an individual B cell receptor (BCR)[1] is specific (2, 4–6). Endocytosis via the BCR not only greatly increases the sensitivity of processing but also helps ensure that B cells stimulate only T cells that are specific for the processed Ag (2, 4–9).

BCR–Ag complexes first enter early endosomes via clathrin-coated vesicles. A fraction of these complexes return, within recycling endosomes, to the surface, while another fraction is targeted to late endosomes and lysosomes

where the majority of Ag is degraded to amino acids (8, 9). MHC class II molecules are contained throughout these endocytic compartments in varying quantities. This broad distribution of class II and associated molecules suggests that peptide–class II complexes can be formed at multiple sites, collectively termed MIIC (MHC-containing compartments; 1–3). However, intracellular accumulation of immunogenic complexes may not reflect physiologically relevant sites of Ag processing or peptide loading. For example, immature dendritic cells form peptide–class II complexes in late endosomes or lysosomes, but few of these complexes are recruited to the plasma membrane (3). A related issue is whether APCs contain specialized compartments for Ag processing. Cell fractionation has revealed the existence of distinct endosome-related class II vesicles that may serve this function (9, 10).

To dissect the relative contributions of various endocytic compartments to Ag processing, we have used a novel strategy using rab proteins. Monomeric GTPases of the rab protein family have proven useful as tools for studying the functions of vesicular traffic in a variety of cell types (12). Rabs are believed to assist in the assembly of large protein

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[1]*Abbreviations used in this paper:* BCR, B cell receptor; DNP, dinitrophenyl; HRP, horseradish peroxidase; TfnR, transferrin receptor; WT, wild-type.

complexes needed for vesicle–vesicle fusion (12). Since every family member has a characteristic organelle distribution, expression of mutant rab proteins can selectively target individual membrane transport steps (12–15). For the early endocytic pathway, rab5 appears to regulate entry of incoming clathrin-coated vesicles into early endosomes, whereas rab4 and rab11 assist in later steps of receptor recycling (13–15). By expressing a dominant-negative mutant of rab4, we demonstrate a critical role for early endocytic compartments in receptor-mediated, but not nonspecific, Ag processing.

Materials and Methods

Establishment of Stable Transfectants. A6B9 is a stable cell line derived from the IIA1.6 B cell lymphoma, a cell that lacks endogenous FcγRII-B1 expression, transfected with a pCB6 expression vector (G418-resistance) containing the cDNA for FcγRII-B2 (16). For expression of rab4, A6B9 cells were electroporated (Bio-Rad Gene Pulser, 300 volts and 960 μF; Bio-Rad, Hercules, CA) with the plasmid pMCFRpac (puromycin resistance) containing an EcoRI fragment derived from the human rab4a cDNA (15). The pMCFR vector, provided by Tom Novack and Lisa Denzin (Yale University, New Haven, CT), provides high expression levels in lymphocytes (17). The wild-type (WT) rab4 was tagged for Ab detection with an epitope from influenza virus (HA) to distinguish the transfected from endogenous proteins. Epitope tagging has been shown to affect neither the localization nor function of rab4 (17). Drug-resistant cells were screened initially for rab4 expression by Western blotting. Expression was verified routinely by flow cytometry (FcγRII-B2) or immunofluorescence microscopy and Western blotting (rab4). Clonal populations were maintained as stable lines for these studies; typically 80–90% of each cell line coexpressed the transfected genes. Cells were grown in IMDM with 10% FCS, 50 μM β-mercaptoethanol, 600 μg/ml geneticin (GIBCO BRL, Gaithersburg, MD), and 2.5 μg/ml puromycin (Sigma Chemical Co., St. Louis, MO) with 5% CO_2 at 37°C.

Western Blotting. Cell pellets were lysed in 0.5% Triton X-100 detergent and clarified. Protein was quantitated with the Bio-Rad protein assay and normalized. Laemmli buffer–solubilized cell lysates (10 μg) were run on 14% polyacrylamide SDS-gels and transferred to nitrocellulose using a carbonate buffer system as previously described (15). Western blot analysis using rabbit serum specific for a peptide sequence derived from the COOH-tail of rab4 (8091) or a glutathione *S*-transferase–modified rab4 protein and chemiluminescent detection (Pierce Chemical Co., Rockford, IL) was followed by densitometric scanning of the fluorographs.

Flow Cytometry. After washing in PBS without Ca^{2+} or Mg^{2+}, cells were incubated with fluorescent Abs (1 μg Ab/10^6 cells; PharMingen, San Diego, CA) in PBS with 2% BSA, 0.5% sodium azide on ice for 1 h. After labeling, cells were washed and fixed in 1% paraformaldehyde for flow analysis. Nonspecific binding was determined using purified rat IgG, isotype-specific Abs, or secondary Ab alone; nonspecific binding to the FcR was blocked with 2.4G2. A FACSVantage® (Becton Dickinson, San Jose, CA) was used to visualize cell surface fluorescent labeling. Data analysis was performed using CellQuest software (Becton Dickinson). MKD6-PE was a generous gift from Marilyn Kehrey at Boehringer Ingelheim Pharmaceuticals, Inc.

Confocal Microscopy. Log phase B cells were harvested, washed with PBS^{2+}, and then allowed to settle on Alcian blue-coated coverslips. Cells were fixed in 3.5% paraformaldehyde in PBS, quenched with 20 mM glycine, and blocked/permeabilized in IMDM containing 10% FCS, 10 mM Hepes, pH 7.4, and 0.02% saponin. Primary Abs used to visualize each marker were as follows: for rab4, an affinity purified polyclonal 8091; for lgpA, supernatants of the GL2A7 mAb; for MHC class II, the M5114 mAb; and for transferrin receptor (TfnR), the TIB219 mAb. Optical sections (0.2 μm) were viewed using a microscope (MRC 650; Bio-Rad) with an ×63 objective and a magnification of 1.5.

Endocytosis and Degradation. Endocytosis assays were performed as previously detailed (17, 18). In brief, cells were incubated with Ags (10^7 cells/ml) for 1 h at 0°C. After multiple washes, cells were warmed to 37°C for various chase times, cooled again and the cell- and media-associated Ags were quantified by assaying the horseradish peroxidase (HRP) activity in the presence of Triton X-100. In all cases, immune complexes were formed by incubating (30 min at 37°C) affinity pure anti-dinitrophenyl (DNP) polyclonal serum with DNP-modified chicken OVA (DNP-OVA). In all experiments immune complexes were composed of Ab–Ag complexes with a molar ratio of 2.5:1, respectively. HRP was assayed using *o*-phenylenediamine dihydrochloride (Sigma Chemical Co.) as substrate. DNP-modifications of OVA, HRP, and BSA were as previously described (17). DNP-BSA was iodinated as previously described (18).

Ag Presentation. Presentation assays comprised an incubation of B cells (10^5 cells/well), T cell hybridomas (10^5 cells/well), and varying concentrations of Ag in 96-well dishes at 37°C with 5% CO_2 overnight. Supernatants were assayed for the presence of IL-2 using either a [^{3}H]thymidine proliferation assay with an IL-2–dependent CTLL-2 cell line (42, 50) or an IL-2 ELISA (PharMingen). The 2R.50 T cell hybridoma was a gift from Rick Mitchell (Harvard Medical School, Cambridge, MA) (4, 11); DO-11.10 T cell hybridoma was provided by Philippa Marrack (Colorado University, Denver, CO) (19). In the case of presentation using fixed APCs, B cells were fed Ag for 3–4 h, fixed in 0.5% paraformaldehyde at room temperature for 20–30 min, and washed extensively with 20 mM glycine before incubation with T cell hybridomas (4).

Results

Production of B Cell Lines Expressing WT and Mutant rab4. For A20 B cells, membrane fractionation studies suggest that only early endosomal compartments are required for BCR-dependent processing (9–11), consistent with observations that relatively little class II accumulates at later endocytic sites (17). To study the role of rab4 and the recycling pathway in B cell presentation, we produced stable transfectants of mouse B cells overexpressing either a WT rab4 protein or a dominant negative mutant form, rab4N^{121}I, which contains an asparagine to isoleucine point mutation known to inhibit nucleotide binding (15). Stable cell lines were produced using A6B9, a derivative of A20 cells transfected with an internalization-competent isoform of the mouse macrophage Fc receptor II (FcγRII-B2), thus allowing for endocytic uptake of antigenic immune complexes (16). WT rab4 and rab4N^{121}I were expressed at levels 20- and 8-fold, respectively, above the endogenous protein levels (Fig. 1 *A*).

We first analyzed the cell surface expression of several membrane proteins involved in Ag presentation. Flow cy-
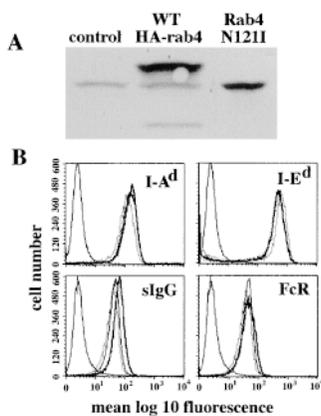
Figure 1. Overexpression of HA-tagged WT rab4 or dominant negative rab4N[121]I did not affect MHC class II expression. (*A*) Western blots representing cell lysates derived from stable cell lines expressing control (vector alone), WT, and rab4N[121]I are shown. The predominant protein that reacted with rab4-specific antisera corresponded to a MW of ~34 kD. Note the slower mobility of the WT protein as expected due to the HA-tag. The lowest molecular weight band could be eliminated by including protease inhibitors in the lysis buffer. (*B*) Flow cytometric scans of cells labeled for I-A[d] (MKD6-PE); I-E[d] (14-4-4S-FITC); FcR (2.4G2 and an FITC-labeled goat anti–rat IgG Fab fragment); or membrane IgG (rabbit anti-IgG2a and FITC-goat F(ab')₂ anti–rabbit IgG). Each graph represents the overlay of traces from control (*thin line*), WT (*thick line*) and rab4N[121]I (*dotted line*) cell lines and includes an overlay of the nonspecific binding.

Figure 2. WT and mutant rab4 are restricted to early endocytic compartments in transfected A20 B cells. Scanning laser confocal micrographs show the intracellular distributions of WT and rab4N[121]I relative to TfnR (*A*), the lysosomal membrane glycoprotein lgpB/lamp-2 (*B*), and MHC class II (I-A[d], *C*).

tometry showed that both of the MHC class II alleles, I-A[d] and I-E[d], were unaffected (Fig. 1 *B*) by overexpression of either WT rab4 or rab4N[121]I. In pulse–chase studies, we saw no effect on the synthesis of MHC class II molecules or formation of SDS stable α/β dimers (20 and data not shown), suggesting that peptide loading of some Ags was normal in the stable lines. Additionally, the levels of surface expression of both BCR and FcRIIγ-B2 were similar in all transfectants (Fig. 1 *B*). Thus, overexpression of either WT or mutant rab4 had no major effect on the surface expression of several functional membrane proteins.

We next determined the intracellular distribution of several different endosomal markers relative to that of WT or mutant rab4 using confocal microscopy. Immunofluorescent labeling of saponin-permeabilized cells for MHC class II, lgp-A/lamp-1, and TfnR showed no dramatic differences between the control, WT, and rab4N[121]I cells as found in other cell types (15, 21). Rab4 itself appeared as small punctate structures whose labeling showed partial overlap with Tfn, a recycling endosomal marker (15, 21). As expected, neither the WT nor mutant rab4s colocalize with lgp-A, indicating that their distribution did not include late endosomes or lysosomes (Fig. 2 *B*). Only a minor degree of colocalization between rab4 and MHC class II molecules was observed.

Expression of Mutant rab4 Does Not Affect Ag Uptake and Degradation. Identifying which elements of the endocytic pathway are most important in BCR-mediated Ag processing has proved a vexing problem. There is evidence that different compartments are involved, depending upon the type of Ag or epitope; however, many of these studies have relied upon Ag delivery via nonspecific fluid phase endocytosis (1–3). To address this issue we studied the ability of these stable lines to process receptor-bound Ags. We first monitored the ability of the rab4-transfected cells to internalize and degrade different Ags. To measure BCR-mediated endocytosis, the HRP-conjugated rabbit F(ab')₂ anti–
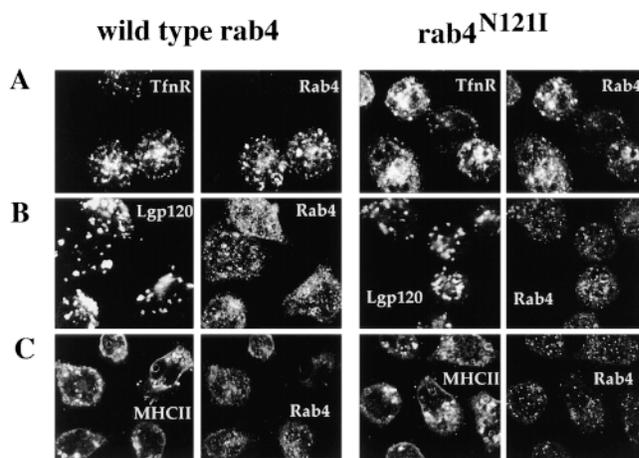
mouse Ab was bound to the cell surface in the cold, as Ag, then followed into the cells upon warming by measuring the total versus surface HRP activity. To measure endocytosis of Ag–Ab complexes via transfected FcγRII-B2, the same assays were conducted except using immune complexes composed of HRP and anti-HRP IgG. Expression of either WT rab4 or rab4N[121]I did not significantly alter the rates of internalization of BCR-bound F(ab')₂ fragments relative to mock-transfected control cells (Fig. 3 *A*). Similar results were obtained for FcR-bound immune complexes (Fig. 3 *B*).

Internalized Ag also appeared to be degraded at normal rates as determined by measuring the release of TCA-solu-
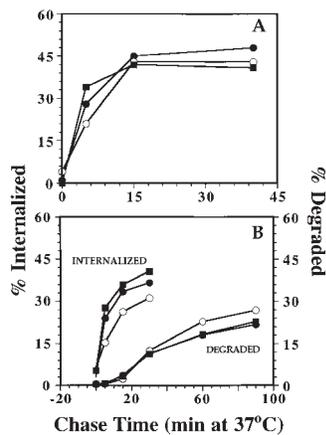

Figure 3. Expression of WT and rab4N[121]I had no effect on receptor-mediated Ag endocytosis and degradation. (*A*) Endocytosis of rabbit F(ab')₂ anti–mouse IgG by BCR. A20 cells were incubated with HRP-rabbit F(ab')₂ anti–mouse IgG (50 μg/ml) in the cold for 1 h, washed, and then rapidly warmed to 37°C for 0–45 min. Surface versus total F(ab')₂ fragments were defined as the HRP activity in the absence or presence of Triton X-100, respectively (47). The percentage of intracellular ligand was estimated by subtraction from the total and plotted against time of uptake. (*B*) Internalization and degradation of Fc receptor-bound Ag. FcR-mediated endocytosis was determined using HRP–rabbit anti-HRP immune complexes similar to the assay described in *A*. Degradation of FcR-bound ligands was determined in separate experiments using immune complexes formed from iodinated BSA and affinity purified anti-DNP IgG. After each chase time, the appearance of TCA-soluble ^{125}I in the medium was determined.

ble ^{125}I into the medium after uptake of immune complexes containing radioiodinated DNP-BSA and anti-DNP IgG. We chose to follow the fate of DNP-BSA since the time course of degradation is known to be more rapid than that of either the Ig Ag or HRP molecule; OVA could not be efficiently iodinated. As shown in Fig. 3 B, the degradation rates of Ag were not affected significantly by rab4 WT or rab4N[121]I. Thus, overexpression of either WT or mutant rab4 had little if any effect on the receptor-mediated uptake or degradation of bound Ag.

Expression of rab4 N[121]I Selectively Blocks FcR-mediated Ag Processing. In the absence of any obvious alterations in the endocytosis or lysosomal delivery of Ag via BCR or FcR, it seemed likely that the expression of WT or mutant rab4 would similarly have no effect upon Ag processing and presentation. However, we found that expression of the dominant negative rab4 N[121]I mutant significantly inhibited the processing of several different Ags.

We first tested the ability of the transfected A20 cells to process and present OVA to the DO.11.10 T cell hybridoma. Immune complexes of DNP-OVA and rabbit anti-DNP IgG (16, 18) were incubated overnight together with the Ag-specific T cell hybridoma and control B cells (expressing FcγRII-B2); FcR-expressing B cells transfected with WT rab4 or rab4N[121]I; or the FcR-negative IIA1.6 B cell. As shown in Fig. 4 A, cells overexpressing WT rab4 exhibited processing and presentation activities, as measured by IL-2 quantities, only slightly lower than control cells containing solely endogenous rab4. Maximum presentation was reached at ~1 μg/ml OVA. Most interestingly, rab4N[121]I-expressing cells were negative for presentation of

the Ag concentrations used (0.1–2 μg/ml), and were almost indistinguishable from the parental IIA1.6 cells.

Remarkably, this defect in Ag processing was selective for receptor-mediated Ag internalization. Rab4N[121]I-expressing cells showed no significant differences in their presentation capacity when incubated with soluble OVA relative to control cells or WT rab4 overexpressors (Fig. 4 B). Importantly, the concentrations of DNP-OVA required for presentation as soluble Ag were 10³-fold higher than that for FcR-bound DNP-OVA. Similar results were obtained whether T cell stimulation was monitored using [³H]thymidine incorporation or IL-2 release assays (data not shown). Demonstration that the defect was restricted to Ag processing, as opposed to Ag presentation, was illustrated by the observation that addition of a synthetic OVA peptide corresponding to the DO-11.10 epitope (OVA 323–339; reference 19) produced indistinguishable T cell responses in all transfected APCs (Fig. 4 C). This result was consistent with the similar amounts of surface MHC class II found on all cell lines (Fig. 1 B).

These results were then extended to a second FcR-internalized Ag. Immune complexes of the cI fragment of λ repressor were generated by incubating the Ag together with two different cI fragment–specific mAbs. Studies have shown previously that these immune complexes can be processed and presented to the 24.4 T cell hybridoma (16). Overexpression of WT rab4 had little effect on the processing and presentation of the cI-containing immune complexes; however, rab4N[121]I expression almost completely blocked cI processing (Fig. 5 A). Similar to OVA, the block seemed to be at the level of Ag processing since all cell types equally presented the synthetic peptide corresponding to the 24.4 T cell epitope (residues 12–26; Fig. 5 B) as well as the intact cI fragment 1–102 (data not shown). In the experiments shown, the IL-2 responses reached for immune-complexed Ag were suboptimal (Fig. 5 A) owing to the variability in T cell hybridoma response, and are not a reflection of the highest 24.4 T cell response that we have observed for this Ag.
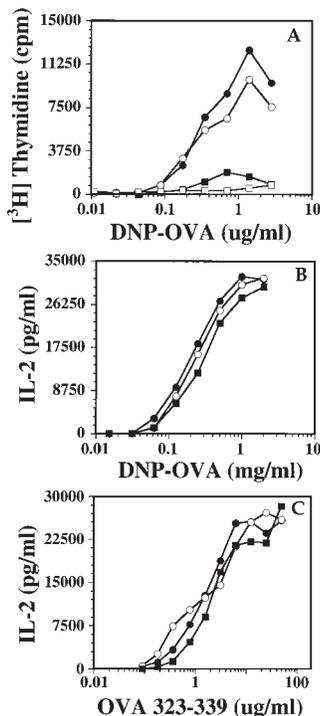

Figure 4. Expression of rab4N[121]I blocks presentation of Fc receptor-bound DNP-OVA, but not soluble DNP-OVA or an immunogenic OVA peptide. Graphs illustrate the presentation of increasing concentrations of DNP-OVA either as an immune complex (*A*) or free protein (*B*) by FcR-negative A20 B cells transfected with FcγRII-B2 and the indicated rab protein to the OVA-specific DO-11.10 T cell hybridoma. Similar conditions were done with immunogenic OVA323-339 peptide (*C*). Secreted IL-2 was measured by ELISA or [³H]thymidine incorporation. Symbols represent control mock-transfected for rab4 cells (●), WT rab4 cells (○), rab4N[121]I expressors (■), and the FcR-deficient IIA1.6 cell line (□). Note that the range of Ag and peptide concentrations varies by 10³-fold in each of the experiments shown.
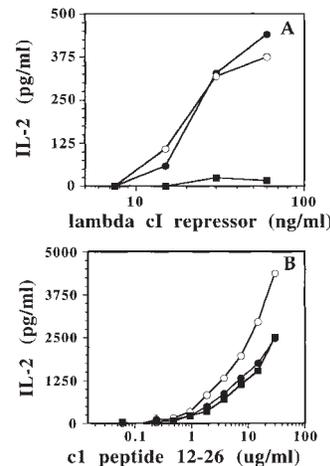

Figure 5. Expression of mutant rab4 blocks presentation of Fc receptor-bound λ repressor but not soluble λ represssor. FcR-negative A20 B cells transfected with FcγRII-B2 and the indicated rab protein tested for their presentation to the λ repressor-cI fragment-specific 24.4 T cell hybridoma with the cI fragment as an immune complex (*A*) or synthetic peptide (residues 12–26) (*B*) as in Fig. 3.

Rab4 Is also Required for Presentation of BCR-bound Ag.
Although the Ag specificity of the endogenous BCR in A20 cells is unknown, the cells can process and present an epitope found in rabbit IgG after internalization of rabbit anti–mouse Ig (4). Control, rab4 WT, and rab4N[121]I-expressing cells were incubated with various concentrations of an $F(ab')_2$ rabbit anti–mouse IgG (0.001–1 mg/ml) and the 2R.50 T cell hybridoma for 18–24 h and then the supernatants were tested for IL-2. The expression of the rab4N[121]I mutant significantly depressed the processing and presentation of the rabbit IgG epitope (Fig. 6), shifting the dose–response curve by >10-fold. Since the precise epitope recognized by 2R.50 T cells is unknown, it was not possible to confirm directly that the defect was limited to Ag processing as for cI and OVA. However, given the results obtained for cI and OVA, as well as the observation that normal rates and quantities of $F(ab')_2$ rabbit anti–mouse IgG were internalized and degraded by cells expressing the rab4 mutant, our data strongly suggest that the block in the presentation of this BCR-bound Ag was at the level of processing. Since the A20-cell line can be stimulated to secrete its own IL-2 through the binding of both monovalent and divalent Abs to the BCR (20) we confirmed these results using fixed Ag-fed APCs (data not shown).

The phenotype of the rab4N[121]I-expressing A20 cells demonstrates that although endocytosis is necessary for Ag processing, the mere fact that an Ag is internalized is insufficient to ensure a functional presentation event. These cells take up and degrade Ag at normal rates when internalized by either the BCR or by a transfected, endocytosis-competent FcR. Yet their ability to process at least three different Ags was dramatically reduced. The defect could not be explained by an inability of the rab4N[121]I-expressors to present Ag, since they were completely capable of stimulating T cells after capturing synthetic immunogenic peptides. Even more remarkably, they remained capable of processing and presenting the very same Ags if internalized as fluid phase components, albeit at >10^3-fold higher concentrations.

Discussion

The humoral immune response is dependent critically on the ability of B cells to present Ags only to those T cells that share their Ag specificity. Thus, B cells can present Ags internalized by the BCR >10^3-fold more efficiently than Ags internalized nonspecifically by fluid phase endocytosis (2, 4–6). B cells appear similarly efficient at presenting virtually any Ag if internalized as an immune complex, probably explaining why B cells normally express an endocytosis-defective splice isoform of FcγRII (10). Our results suggest that B cells may possess at least one additional feature that helps ensure the efficiency and, therefore, specificity of receptor-mediated Ag processing; namely the existence of a rab4-dependent step that is required for the presentation of receptor-bound, but not nonspecifically internalized, Ags.
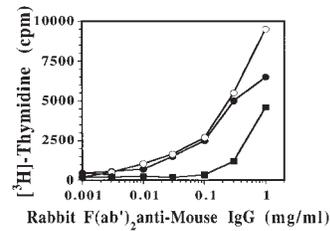

Figure 6. Expression of rab4N[121]I blocks presentation of rabbit IgG bound to endogenous BCR on A20 cells. A20 cells were incubated with the indicated concentrations of $F(ab')_2$ fragments of an affinity-purified rabbit anti–mouse IgG Ab (Cappel, Durham, NC) together with the rabbit IgG-specific T cell hybridoma, 2R.50, under the conditions described in Fig. 3. Both mock- and WT rab4–transfected B cells stimulated the T cells at Ag concentrations <10-fold lower than rab4N[121]I-expressing B cells.

One or more explanations might account for the ability of rab4N[121]I expression to selectively inactivate receptor-mediated Ag processing. First, it is possible that receptor-bound and nonspecifically internalized Ags are processed and loaded onto class II molecules at different sites. Thus, expression of rab4N[121]I may prevent delivery of BCR- or FcR-bound Ags, or the delivery of critical Golgi complex–derived components (e.g., MHC class II, HLA-DM, proteases), to a specialized site(s) for receptor-mediated Ag processing. Conceivably, such a site may correspond to the class II vesicle compartment previously identified in A20 cells (9, 10). Second, rab4N[121]I may block delivery of Ag or class II to otherwise conventional endocytic compartments. Third, it is possible that peptide–class II complexes form normally, but are prevented from being routed to the plasma membrane by rab4N[121]I expression. Although these questions would be best addressed by subcellular fractionation, it has thus far proved impossible to adopt this approach. Rab4N[121]I-expression is sufficiently unstable so as to prevent the propagation of sufficient quantities of homogeneously expressing cells to permit fractionation experiments.

Much remains to be learned about the intracellular compartments responsible for Ag processing and peptide loading. Different compartments may be used by APCs depending upon the cell's primary mode of Ag delivery and proteolytic susceptibility, as each organelle is unique in both pH and enzyme content (1–3). Early processing events would rapidly generate a repertoire of class II–peptide complexes from those Ags that require minimal proteolysis, perhaps skewing the immune response towards Ags with characteristically low dependencies on invariant chain or class II–associated molecules such as HLA-DM (22, 25). This is likely true for both OVA and λ repressor cI fragment (21, 24). Indeed, delivery of such Ags to later compartments, richer in proteolytic enzymes, might result in the loss of such early epitopes due to degradation. Although we have not yet been able to define the precise step or pathway affected by expression of mutant rab4, our results demonstrate that the early endosome-recycling pathway, rather than the late endosome-lysosome pathway, plays a disproportionately important role in the productive processing of at least several receptor-bound Ags.

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