Endosomal Targeting by the Cytoplasmic Tail of Membrane Immunoglobulin

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Membrane-bound immunoglobulin (mIg) of the IgG, IgA, and IgE classes have conserved cytoplasmic tails. To investigate the function of these tails, a B cell line was transfected with truncated or mutated γ2a heavy chains. Transport to the endosomal compartment of antigen bound by the B cell antigen receptor did not occur in the absence of the cytoplasmic tail; and one or two mutations, respectively, in the Tyr-X-X-Met motif of the tail partially or completely interrupted the process. Experiments with chimeric antigen receptors confirmed these findings. Thus, a role for the cytoplasmic tail of mIg heavy chains in endosomal targeting of antigen is revealed.

The B cell antigen receptor (BCR) is a multiprotein complex that includes the membrane-bound immunoglobulin molecule (mIg) and the Ig-α,Ig-β heterodimer (*1*). The latter molecules function as the signaling subunit of the BCR. They are also required for the intracellular transport of IgM-BCR to the endosomal compartment, where the bound antigen is proteolytically degraded (*2*). All classes of mIg are associated with the Ig-α,Ig-β heterodimer (*3*), but the heavy chains differ in the length of their cytoplasmic tails: there are 3 amino acids for μm and δm tails and 28 amino acids for γm and εm tails. No function has so far been attributed to the conserved cytoplasmic sequence of mIgG molecules that are expressed on memory B cells.

To analyze the function of the 28–amino acid cytoplasmic tail of the γ2am heavy chain, we truncated or mutated the sequence (*4*) coding for this tail in the expression vector pSV2neoγ2am (*5*). The chain lacking all cytoplasmic amino acids except for the three KVK (*6*) residues (which are identical to the COOH-terminus of the μm chain) we called γ2amtl. Point mutations were introduced to change the YXXM motif in the γ2am cytoplasmic sequence to either LXXM (γ2amY20L) or LXXL (γ2amY20L,M23L). Expression vectors for these heavy chains were transfected into K46λ12 B lymphoma cells expressing a λ1 light chain. The expressed wild-type and mutated γ2am chains associate with the λ1 light chain to form 5-iodo-4-hydroxy-3-nitrophenyl-acetyl (NIP)–specific mIgG2a molecules.

After surface biotinylation of K46λγ2am and K46λγ2amtl cells, the wild-type and tailless IgG2a-BCR complexes were affinity-purified over NIP-Sepharose (*7*) and analyzed by protein immunoblotting (Fig. 1). This analysis confirmed that the γ2amtl chain has a lower molecular weight than the wild-type γ2am chain (Fig. 1, lanes 4 and 2) and showed that both mIgG2a molecules are associated with the Ig-α,Ig-β heterodimer to the same extent. Yet unidentified surface proteins of 41 and 42 kD were copurified together with the wild-type but not truncated IgG2a-BCR complex. These molecules may thus require the γ2am tail for efficient binding. A fluorescence-activated cell sorter analysis confirmed that similar amounts of mIgG2atl and wild-type mIgG2a were expressed on K46 cells, whereas the two point-mutated mIgG2a molecules were expressed in amounts that were reduced by a factor of 3 to 5.

The endosomal transport of antigen bound to wild-type or mutated IgG2a-BCR was tested in an ovalbumin (OVA) peptide presentation assay (*8*). The different γ2am transfectants of K46λ12 cells were cocultured with the T helper cell line 3DO54.8, which is specific for the OVA 323-339 peptide in the presence of NIP-OVA or OVA alone (Fig. 2). The K46λγ2am cells, which express wild-type IgG2a-BCR, were able to present the antigenic peptide to the T cells when exposed to low amounts of NIP-OVA, whereas exposure to the same amount of OVA did not result in antigen presentation (Fig. 2B; $P < 0.001$). K46λγ2amtl cells, which express the tailless IgG2atl-BCR complex, did not present the OVA peptide even when cultured with large amounts of the specific antigen (Fig. 2C). The same defect was found in two independent γ2am transfectants of K46λ12 that expressed an IgG2a-BCR with a double (Y → L, M → L) (*6*) mutation of the YXXM motif. These are referred to as K46λγ2amY20L,M23L (Fig. 2, E and F). K46λγ2amY20L cells, expressing an IgG2a-BCR with a single Y → L mutation of the YXXM motif, had a modest but not statistically significant capacity to present antigen (Fig. 2D; $P < 0.3$). The

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were amplified individually or in groups from genomic DNA by means of primers designed from intron sequences (supplementary PCR primer and ddF primer sequences can be found at www.sciencemag.org or www.nhgri.nih.gov). PCR was performed in 25-μl reactions containing 100 ng of DNA and 0.5 U of AmpliTaq Gold (Perkin-Elmer) according to the manufacturer's protocol. Dimethyl sulfoxide (final concentration 5%) was included for exons 2, 9, and 10. The primary PCR products were subjected to a dideoxy chain termination reaction with 200 μM dideoxyguanosine triphosphate (Boehringer Mannheim) and AmpliTaq Gold, as described [G. H. Sarkar, H. J. Yoon, S. S. Sommer, *Genomics* **13**, 441 (1992)], with modified gel running conditions. The ddF reactions were diluted 1:4 in buffer containing 7 M urea, 50% formamide, bromophenol blue, and xylene cyanol. Reactions were heated at 94°C for 5 min and chilled on ice, and a 5-μl sample was loaded on a nondenaturing gel [0.75× Mutation Detection Enhancement (MDE) (FMC Bioproducts, Rockland, ME) in 0.5× tris-borate EDTA (TBE)] on a sequencing apparatus. The gel was electrophoresed at a constant power of 8 W at room temperature in a buffer system (consisting of 0.5× TBE in the top reservoir and 0.8× TBE with 0.5 M sodium acetate in the bottom reservoir) until the bromophenol blue reached the bottom of the gel. The gel was removed on Whatman paper, dried for 30 min in a sequencing gel drier, and autoradiographed overnight. One ddF primer could screen about 250 bp; if the region to be screened in the primary PCR product was larger, additional primers were used for ddF. Samples showing changes in band patterns were subjected to cycle sequencing with the same primary PCR product and the same end-labeled primer as was used in the ddF reaction. For insertion or deletion type changes in which the actual bases involved could not be ascertained from the sequence of the heterozygous patient sample, the primary PCR product was cloned in the TA cloning vector pCRII (Invitrogen) and then sequenced.

21. Confirmation that the mutation segregated with MEN1 was achieved by direct sequencing of PCR products from other affected family members. Independent confirmation of the sequence change in affected individuals was achieved by restriction digestion of the appropriate exon PCR product for 512delC (creates an Afl II site), W436R (creates Msp I and Nci I sites), and R527X (creates a Bsu 36I site). For the remainder, analysis was carried out with radioactively labeled allele-specific 16- to 20-nucleotide oligomers, corresponding to the wild-type or mutant sequence, that were hybridized to slot blots of exon PCR products as described [J. Lyons *et al.*, *Science* **249**, 655 (1990)].
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28. This paper is dedicated to the memory of Gerald D. Aurbach. We thank all the MEN1 families who participated and the clinicians (NIDDK–National Institute of Child Health and Human Development NIH Interinstitute Endocrine Training Program, NCI Surgery Branch, and Clinical Center Diagnostic Radiology Department) who helped care for them. We thank C. Cummings, N. Dietrich, L. Gieser, B. Pike, C. Robbins, and S. Saggar for technical support, S. Sommer for advice on the ddF procedure, D. Leja for assistance in preparing the illustrations, and P. Fakunding for manuscript preparation. Supported by the intramural research programs of NHGRI, NIDDK, NCI, and NLM, the Fritz Thyssen Stiftung Fund (C.H.), and a U.S. Department of Energy Graduate Fellowship (J.S.C.).

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endosomal targeting function of the γ2am tail was tested by a second approach that used expression vectors for chimeric single-chain antigen receptors (scAgR) (9). These receptors consist of the covalently linked V_H and V_L domains of the NIP-specific antibody B1-8 (*10*) attached to the CD8α hinge region and the transmembrane part of the T cell receptor ζ chain, to which different tail sequences can be appended. K46 cells were transfected with several variants of scAgR: one with a wild-type γ2am tail, one with a YXXM point mutation, and one with a truncated (tl) scAgR, which carried RRIDP (6) as a cytoplasmic tail. These were tested in the antigen presentation assay. After exposure to the antigen NIP-OVA, the K46scγ2am cells efficiently presented the OVA peptide (Fig. 3A; $P < 0.001$), whereas K46scγ2amY20L,M23L (Fig. 3C) or K46sctl (Fig. 3D) cells did not. The K46scγ2amY20L cells, which express a chimeric receptor with the Y → L point mutation of the YXXM motif, were able to present antigen, although they did so less efficiently than K46scγ2am cells (Fig. 3B; $P < 0.01$).

It has previously been shown that chimeric receptor molecules containing the cytoplasmic component of Ig-β or Ig-α can mediate transport to the antigen-processing compartment (*2*, *11*). We have generated transfectants of K46 expressing scAgR with either the wild-type or an altered Ig-α tail sequence carrying a Y → F (M1) mutation (*12*) of both tyrosines of the immunoreceptor tyrosine-based activation motif (ITAM). Upon exposure to low doses of NIP-OVA, both transfectants were able to efficiently present the OVA peptide (Fig. 3, E and F; $P < 0.001$). These results showed that the Ig-α tail has a targeting function that is not abolished after a Y → F mutation of the two tyrosines of the ITAM.

The aggregation of chimeric receptors that contain a γ2am tail does not result in intracellular signaling (*2*, *13*), which indicates that the internalization and presentation of antigen are independent from signal transduction. The sequence around the YXXM motif (SPDYRNMIG) (6) is conserved in all IgG classes and has similarities to the NPXY (6) internalization signal of the low-density lipoprotein receptor for which a type I β-turn structure has been demonstrated (*14*). Such a structure seems to mediate the contact between the receptor and intracellular transporter molecules. The tail of the εm chain carries, at the same position as the YXXM motif, a YXXI sequence that may function as an endosomal transport signal for the mIgE molecule.

The targeting function of the YXXM motif is abolished by the double mutation (Y → L, M → L) but not by a single Y → L mutation. Apparently, a bulky hydrophobic amino acid such as leucine can functionally replace a tyrosine in such a targeting signal. It is possible that the YXXL/I sequences in the ITAM of Ig-α and Ig-β also act as targeting signals and that the studied Y → F mutations of the two tyrosines of ITAM (Fig. 3F) are not sufficient to abolish this targeting function. For the FcγRIII receptor, the ITAM in the γ chain has been shown to function as a targeting signal (*15*).

Our data apparently contradict previous studies of a chimeric IgM-MutB-γ2bm molecule that is not targeted to the endosomal compartment (*2*). However, in contrast to scAgR-γ2am, the MutB-γ2bm molecule is only very inefficiently internalized and this may prevent the γ2bm tail from exerting its targeting function. The tailless mIgG2atl has the same cytoplasmic sequence (KVK) as mIgM. Both molecules are expressed on the cell surface in association with the Ig-α,Ig-β heterodimer. It is, however, possible that because of differences in the transmembrane sequence, the mIgG2a is less tightly associated with the heterodimer than the mIgM or mIgD molecules. If, during endosomal transport (perhaps promoted by endosomal pH changes), the Ig-α,Ig-β heterodimer dissociates from mIgG2a, the latter molecule would require its own cytoplasmic targeting signal to reach the endosomal compartment. The transport of a tailless mIgG2atl molecule would thus not occur. The above scheme

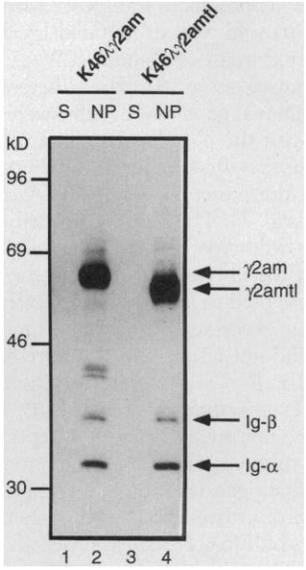

Fig. 1. Composition of wild-type IgG2a-BCR (lane 2) and tailless IgG2atl-BCR (lane 4) on the surface of K46λ12 cells. After surface biotinylation, a 1% digitonin lysate of the different γ2am transfectants was incubated with either Sepharose (S; lanes 1 and 3) or NIP-Sepharose (NP; lanes 2 and 4), and bound molecules were analyzed by SDS-PAGE on 10% gels with protein immunoblotting.

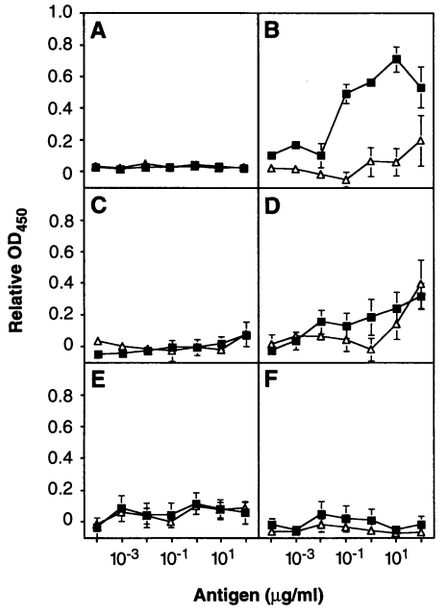

Fig. 2. Dose response of antigen presentation by (**A**) untransfected K46λ12 cells and the different γ2am-transfectants, (**B**) K46λγ2am, (**C**) K46λγ2amtl, (**D**) K46λγ2amY20L, (**E**) K46λγ2amY20L,M23L#1, and (**F**) K46λγ2amY20L,M23L#2 exposed for 24 hours to different concentrations of either NIP-OVA (black squares) or OVA (white triangles). Mean values and standard deviations from triplicates are shown. IL-2 production is presented as relative OD_{450}, as described in (*8*).

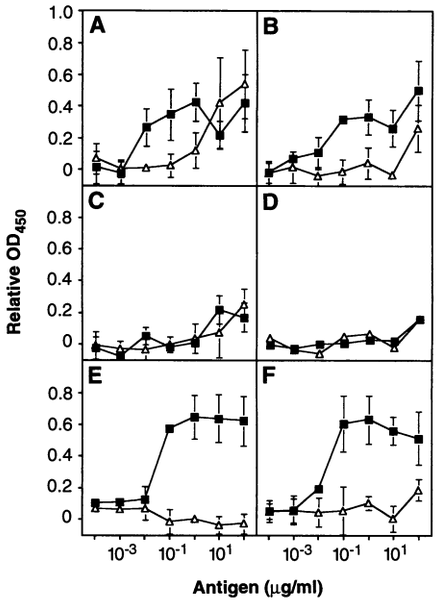

Fig. 3. Dose response of antigen presentation by K46 cells expressing different chimeric scAgRs. The transfectants (**A**) K46scγ2am, (**B**) K46scγ2amY20L, (**C**) K46scγ2amY20L,M23L, (**D**) K46sctl, (**E**) K46sclg-α, and (**F**) K46sclg-αM1 were exposed for 24 hours to different concentrations of either NIP-OVA (black squares) or OVA (white triangles).

could also explain why a mIgM molecule that carries a Y → F mutation in its transmembrane part and presumably is less tightly associated with the Ig-α,Ig-β heterodimer than wild-type mIgM does not reach the antigen-presentation compartment (*16*).

B cells require T cell help for the production and affinity maturation of antibodies against protein antigens (*17*). Our finding that B cells with a tailless IgG2a-BCR cannot efficiently present antigen to T cells explains the reduction in IgG1 and IgE serum concentrations in mutant mice that express tailless IgG1-BCR and IgE-BCR, respectively (*18*). Activated memory B cells appear to require the cytoplasmic tails of mIg molecules and the efficient antigen presentation connected with this structure in order to expand and differentiate into antibody-producing plasma cells. Thus, the conserved COOH-terminal sequences of mIg molecules are finally assigned an important immunological function.

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4. The expression vector pSVneoγ2amtl is a derivative of pSVγ2am. For the construction of this vector we used two overlapping oligonucleotides as a linker to introduce a Bgl II site and a stop codon after the sequence coding for the cytoplasmic amino acids KVK. Point mutations were introduced into the sequence coding for the γ2am tail by the polymerase chain reaction (PCR). As a template for PCR, we used the linearized pγ2am plasmid. The PCR products were cloned into pγ2am from which we generated the expression vectors pSV2neoγ2amY20L and pSV2neoγ2amY20L,M23L. The expression vectors for the chimeric scAgR were constructed from psc-cyt (R. Müller and M. Reth, in preparation), a derivative of the vector pHβAPr1neoFvCD3ζ (*9*). The psc-cyt vector encodes the V_H and V_L domains of the NIP-specific antibody B1-8, followed by a CD8α linker region and the CD3ζ transmembrane part, and carries a Bam HI cloning site after the CD3ζ sequence. Bam HI fragments of the wild-type or mutated cytoplasmic γ2am and mb-1 coding sequences were obtained by PCR and inserted into psc-cyt to yield the expression vectors pANPCD8γ2am, pANPCD8γ2amY20L, pANPCD8γ2amY20L,M23L, pANPCD8mb-1, and pANPCD8mb-1M1. The pANPCD8tl vector encodes a scAgR with a short cytoplasmic sequence of the five amino acids RRIDP. The vectors were linearized with Pvu I and introduced into K46 and K46λ12 cells by electroporation.
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6. Single-letter abbreviations for the amino acid residues are as follows: D, Asp; G, Gly; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; R, Arg; S, Ser; V, Val; X, any amino acid; and Y, Tyr.
7. Transfected cells were surface-biotinylated and lysed with 1% digitonin buffer as described (*5*). After a preclearing step with Sepharose beads, NIP-specific molecules were immunoprecipitated from the lysates with the use of NIP-Sepharose or Sepharose as control. After being washed three times with the 1% digitonin buffer, the NIP-Sepharose beads were boiled in reducing SDS sample buffer. The released proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels and electroblotted onto a nitrocellulose membrane. Biotinylated proteins were detected with Str-HRP and the ECL detection system (Amersham, Braunschweig, Germany).
8. Antigen presentation of K46-transfectants exposed to the antigen NIP-OVA was tested by the ability of the T cell hybridoma 3DO54.8 (which recognizes the OVA peptide 323-339 in the context of the I-A^d major histocompatibility complex class II molecule) to secrete interleukin 2 (IL-2). IL-2 production was quantitated in chromogenic proliferation assay (EZ4U, Biomedica, Vienna) using the IL-2–dependent cell line CTLL. The K46 transfectant and the 3DO54.8 line (4×10^4 cells each) were incubated in a 96-well plate for 24 hours in Dulbecco's modified Eagle's medium (DMEM) with different concentrations of NIP-OVA or OVA. The plates were then frozen at −80°C, and after thawing, the cell-free supernatant of each well was incubated for 24 hours with 3000 IL-2–starved CTLL cells. As high and low controls, CTLL cells were cultured with or without excess of exogenous IL-2. After addition of substrate solution and incubation for 3 to 5 hours, absorbance at 450 nm (OD_{450}) was measured on a Molecular Devices ELISA reader and analyzed with the program Softmax. The calibration of the assay was done by the formula (sample OD_{450} − low control OD_{450})/(high control OD_{450} − low control OD_{450}) and is presented as relative OD_{450}. Mean values and standard deviations from triplicates were then used to plot the dose-response curves. The statistical relevance of each mean value was verificated by use of Student's *t* test. Apart from transfectant K46λγ2amY20L, two or three independent clones of each transfectant were analyzed. B cells were propagated in RPMI 1640 medium containing 10% fetal calf serum (FCS) (Vitromex, Vilshofen, Germany) and G418 (0.5 mg/ml); the T cells were cultured in DMEM containing 10% FCS and, in case of the CTLL cells, 5% of supernatant of the IL-2–producing transfectant X63-IL-2.
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Effect of Transmembrane and Cytoplasmic Domains of IgE on the IgE Response

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B cells use immunoglobulin M (IgM) and IgD as antigen receptors, but after contact with antigen they can switch and use IgG, IgA, or IgE. In mice lacking the transmembrane and cytoplasmic domains of IgE, serum IgE is reduced by more than 95 percent and, after immunization, specific responses are negligible. In mice lacking most of the cytoplasmic tail of IgE, serum IgE levels are reduced by 50 percent and specific responses are reduced by 40 to 80 percent, without a clear secondary response. Thus, membrane expression is indispensable for IgE secretion in vivo, and the cytoplasmic tail influences the degree and quality of the response.

Immunoglobulin E contributes least to the serum immunoglobulins (Igs). Its specific function is not understood, although it is well known as the cause of allergic reactions (*1*). IgE, like other Igs, is also expressed as an integral membrane protein (mIgE) on B cells. The transmembrane segments of mIgs are 25 amino acids long, whereas the cytoplasmic domains vary in size from three residues [Lys-Val-Lys (KVK)] for mIgM and mIgD to 14 to 28 residues for other isotypes (*2*). The nature and effects of the signals generated by mIgs other than IgM and IgD are mostly unknown, but they may control affinity maturation, memory induction, and differentiation into plasma cells (*3*). To study the role of the transmembrane domain and cytoplasmic tail of mIgE, we made mouse lines that carried mutations in these domains in the germline ε gene, using the gene-targeting technique in embryonic stem (ES) cells (*4*, *5*) (Fig. 1). The ΔM1M2 line lacks the transmembrane and cytoplasmic domains of IgE, whereas the KVKΔtail line can only express a cytoplasmic tail of three amino acid residues (KVK), which is identical to the cytoplasmic domain of mIgM and mIgD.

Serum Igs in 7-week-old unprimed mice were measured (Fig. 2A). There was

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