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Production of a Chimeric Form of CD23 That Is Oligomeric and Blocks IgE Binding to the FcεRI[1]

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The low affinity receptor for IgE (FcεRII/CD23) has previously been shown to interact with IgE with a dual affinity. Three chimeric constructs were created containing the lectin domain (amino acids 172–188) or the "neck" and lectin domain (amino acids 157–188) attached to subunits of oligomeric proteins. All chimeras were incapable of interacting with IgE with either a high or low affinity, indicating that the α-helical stalk of CD23 is important for orienting the lectin heads such that an interaction with IgE can occur. This concept received further support in that a chimeric CD23 composed of the human CD23 stalk and the mouse CD23 lectin head bound mouse IgE with a dual affinity, but could only bind rat IgE with a low affinity. Effort was next concentrated on a construct consisting of the entire extracellular (EC) region of CD23. A mutation to the first cleavage site of CD23 (C1M) resulted in a more stable molecule as determined by a decrease of soluble CD23 release. A soluble chimeric EC-C1M was prepared by attaching an isoleucine zipper to the amino terminus (*lz*EC-C1M). The interaction with IgE by *lz*EC-C1M was found to be superior to that seen with EC-CD23. The *lz*EC-C1M could inhibit binding of IgE to both CD23 and the high affinity receptor for IgE, FcεRI, providing further evidence for a strong interaction with IgE. FcεRI inhibition (~70%) was seen at equimolar concentrations of *lz*EC-C1M, implying the effectiveness of this chimera and suggesting its potential therapeutic value. ***The Journal of Immunology*****, 1998, 161: 6696–6704.**

The low affinity receptor for IgE, FcεRII or CD23 (1), is a type II integral membrane protein expressed on murine B cells (2) and follicular dendritic cells (3). Structurally, CD23 consists of a carboxyl terminal lectin head, a stalk consisting of four 21-amino acid (aa)[4] repeat domains, and a transmembrane and cytoplasmic domain (4). Both human (5) and murine (6) CD23 have been found to exist as both a membrane and a soluble protein; the latter is a result of proteolytic cleavage. Through the use of CD23 transgenic (7, 8) and knockout mice (9), this receptor has been implicated in the regulation of Ig production, specifically IgE.

In the past several years, two new ligands for CD23 have been identified. CD21, or CR2, is expressed on B cells, and interactions between these two proteins may enhance homotypic aggregation between B cells (10). In addition, this interaction has been proposed to play a role in IgE regulation (11). More recently, human (12) and murine (13) CD23 have been shown to interact with CD11b/CD18 on monocytes, and this interaction results in inflammatory cytokine release (12, 13). The interaction with these other ligands, especially CD11b/CD18, has shown the involvement of CD23 in immunity through interactions with receptors on both lymphoid and nonlymphoid cells.

Previous work from this laboratory using several different chimeras (14, 15) or a full-size, soluble, recombinant form of CD23 consisting of the entire extracellular region of CD23 (EC-CD23) (15) determined that the stalk of CD23 is important in mediating oligomerization of the protein allowing for a high affinity interaction with IgE via avidity considerations. While EC-CD23 has previously been shown to interact with IgE, albeit with a lower affinity than membrane CD23, a stable form of soluble CD23 (sCD23) that could bind IgE with an affinity closer to that of intact CD23 was still desired. In this regard, several chimeras were made containing stalks that were not subject to proteolytic degradation to further ascertain which domains of CD23 are important in mediating the interaction with IgE. It was found that addition of a trimeric isoleucine zipper to the entire extracellular region of CD23 resulted in a soluble construct that bound IgE in a manner at least equivalent to membrane CD23.

Materials and Methods

Cell culture, Abs, and immunosorbents

African green monkey kidney (COS) cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and used for transient expression of chimeric proteins. COS cells were maintained in supplemented RPMI (Life Technologies (Gaithersburg, MD)) as previously described (15). The Chinese hamster ovary (CHO)-K1 cell line (ATCC) and the $CD23^+$ CHO-K1 cell line, a previously established transfected CHO cell line expressing murine CD23 (14), were maintained as previously described (14). The murine mast cell line C57 (a generous gift from Dr. Stephen Galli, Beth Israel Deaconess Medical Center, Boston, MA) was maintained in Iscove's modified Dulbecco's medium supplemented with

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[4] Abbreviations used in this paper: aa, amino acid; sCD23, soluble CD23; EC-CD23, soluble recombinant CD23 construct consisting of entire extracellular region; MBP, mannose binding protein; MBP-CD23, soluble CD23 chimera consisting of the lectin head of CD23 attached to the leader sequence and stalk of mannose binding protein; CD72-CD23, CD23 chimera consisting of the lectin head of CD23 and the cytoplasmic, transmembrane, and stalk domains of CD72; CD72-CD23neck, CD23 chimera consisting of the same regions of CD72 as in CD72-CD23 attached to the neck and lectin head domains of CD23; human-mouse CD23, CD23 chimera consisting of the lectin head of murine CD23 attached to the cytoplasmic, transmembrane, and stalk domains of human CD23; C1M, cleavage mutant 1; EC-C-CIM, EC-CD23 in which the first protease cleavage site is mutated; *lz*EC-C1M, sCD23 chimera consisting of a modified isoleucine zipper attached to the amino terminus of the entire extracellular domain of cleavage mutant 1; VCU, Virginia Commonwealth University; CHO, Chinese hamster ovary; mIgE, murine IgE; rIgE, rat IgE.

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L-glutamine, Pen/Strep, 10% FBS, and nonessential amino acids (Life Technologies).

Mouse IgE from H1-DNP-ε-26 (16) was purified from ascites as described elsewhere (17), and monoclonal anti-CD23 (B3B4 (6) or 2G8 (7)) was purified from rat ascites. B3B4 and 2G8 are both rat anti-mouse CD23 Abs that interact at a site similar, but not identical, to the site on CD23 that binds IgE. Rabbit B is a polyclonal anti-CD23 Ab purified from rabbit serum, as previously described (7). M15, a mouse IgG1 anti-leucine zipper, was obtained from Immunex (Seattle, WA) (18). B1E3 is a monoclonal rat anti-mouse IgE Ab that was purified from rat ascites (19). Abs were coupled to Affi-Gel-10 from Bio-Rad Laboratories (Richmond, CA) at a concentration of 1 mg/ml. Protein A/protein G Sepharose was purchased from Pierce (Rockford, IL). Recombinant CD40L trimer was a gift from Immunex.

Preparation of chimeric CD23 constructs

The EC-CD23 construct was made and expressed in a eukaryotic expression vector as previously described (15). The EC-CD23 cDNA was moved into the prokaryotic expression vector pET-5a (Novagen, Madison, WI) using PCR to generate a cDNA with a 5′ *Nhe*I (ACTGCTAGCCACTGG GAAACGGAGAA) site and a 3′ *Eco*RI site (TTCACAGAATTCCTCTA GAGTCG). Sequence analysis, using the dideoxy method and an Applied Biosystems (Foster City, CA) 373A Automated DNA Sequencing System performed by the Nucleic Acid Core Laboratory (Virginia Commonwealth University (VCU)/Medical College of Virginia, Richmond, VA), confirmed that the predicted cDNA sequence was correct and that no mutations had been introduced by PCR. The MBP-CD23 chimera, a soluble CD23 chimera, was composed of the lectin head of CD23 attached to the leader sequence and stalk of mannose binding protein (MBP)-C (20) and the lectin domain of CD23. The cDNA fragment encoding the signal sequence and stalk of MBP was generated by PCR using MBP-C in pBSKS (a generous gift of Dr. Kurt Drickamer, Stanford University) with primers that added a 5′ *Hin*dIII site (CATCCGAAGCTTGTGAGGACCATG) and a 3′ *Hin*cII site (CTTCTTTCAACATTTTCACT). The cDNA fragment for the lectin head of CD23 (aa 189 to the end) was generated by PCR using the cDNA for murine CD23 in pCDM8 and the following primers, which added a 5′ *Sma*I site (GATCCCGGGATTTCAAAGGGAACT) and a 3′ *Not*I site (ACAAAGATCCTCTAGAGTCGCGGC). The MBP and CD23 cDNAs were ligated simultaneously into pC-DNA-1-amp that had been digested with *Hin*dIII and *Not*I. Sequence analysis was performed to confirm that the junction between MBP and CD23 was in frame.

The chimeric CD23 protein composed of the cytoplasmic, transmembrane, and stalk domains of CD72 and the lectin head of CD23 (CD72-CD23) was made using a similar PCR-based strategy. The cDNA of CD72 was generated by PCR using CD72 in pBSKS with a 5′ primer (TCACA CAGGAAACAGCTATGAC) and a 3′ primer, which added a *Hpa*I site (AATGTTAACGGAGGACAAGCGCCTCAG) and was cloned directly into the PCR cloning vector PCRII (Invitrogen, San Diego, CA). CD72 in pCRII was digested with *Hin*dIII and *Hpa*I and ligated in frame to the lectin head of CD23 (same fragment as that used with MBP) in pBSKS that had been digested with the same enzymes. The CD72-CD23 cDNA was moved from pBSKS into the eukaryotic expression vector pC-DNA-1-amp via *Hin*dIII and *Not*I digestion. Sequence analysis performed by the Nucleic Acid Core Laboratory (VCU) detected a frame-shift mutation that was corrected using the Chameleon Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Further sequencing analysis confirmed that the final product was free of any mutations. The CD72-CD23neck chimera was made using the same CD72 cDNA as created for the above chimera, cloned in frame to the "neck" and lectin head domain of CD23. The neck domain of CD23 contains aa 157–188. PCR was used to generate the CD23neck cDNA using the following 5′ primer, which added an *Hpa*I site (GGCGT TAACGAGAAGCGCACA) and 3′ primer, which added a 3′ *Not*I site (GAATAGTCGACTCTAGATGCATGCTC). The CD72 and CD23neck cDNAs were cloned in frame after digestion with *Hin*dIII and *Not*I and ligated into the eukaryotic expression vector pCDNA-3 (Invitrogen). Final sequence analysis determined that the final product was free of PCR-induced mutations.

The CD23 chimera consisting of the lectin head of murine CD23 attached to the cytoplasmic, transmembrane, and stalk domains of human CD23 (human-mouse CD23) was made as previously described (14). To analyze the ability of this chimera to bind rat IgE (rIgE), the cDNA was moved from pCDM8 into the high expression vector pEF-BOS (21). The cDNA of human-mouse CD23 was cloned into pEF-BOS after digestion with *Xba*I, and clones containing the insert in the correct orientation were used in the analysis.

Preparation of protease resistant and isoleucine zipper chimeric constructs

The first cleavage site of CD23 was mutated from an asparagine to an alanine to create cleavage mutant 1 (C1M) using the megaprimer PCR-based mutational strategy (22) with the following primers: 5′ (ATC GATATGGAAGAAAATGAATAC) and 3′ (CTTGCAAGGCTTGTGA CATCT). The cDNA product was used in a subsequent PCR reaction as the 5′ primer along with the 3′ primer (GTCGACTCAGGGTTCACTTTTT) to generate the C1M cDNA, which was directly cloned into pCRII. The cDNA was further modified by PCR to create a 5′ *Bam*HI site (CGG GATCCATGGAAGAAAATCAAT) and a 3′ *Eco*RI site (GGAATTCT CAGGGTTCACTTTTTGG), allowing direct cloning into the PCR cloning vector pCRII. Subsequent digestion with *Eco*RI allowed the cDNA to be cloned into pC-DNA-3 (Invitrogen). Sequence analysis determined that the final product was free of PCR-induced mutations. A stable C1M cell line was created by electroporating CHO-K1 cells with C1M in pC-DNA-3 and using neomycin to select for positive clones. Electroporation was performed as previously described (23). CD23 surface expression was monitored by FACS analysis.

The *lz*EC-C1M construct contained the same sequence of cDNA used to create the full-sized sCD23 (EC-CD23 (15)) with the following exceptions: 1) the first cleavage site in CD23 was mutated from an asparagine to an alanine, and 2) a modified leucine zipper motif was added to the amino terminus. The cDNA encoding the leader peptide from the FcεRIα leader (24) was used as with EC-CD23 (15) utilizing the *Hpa*I site to clone the cDNAs together in frame. The sequence for the modified leucine zipper, *lz*, was graciously provided by Dr. Melanie Spriggs at Immunex (25). The cDNA of the *lz* was synthesized such that both *Hin*dIII and *Hpa*I sites were present at the 5′ end and an *Eco*RI site was present at the 3′ end, allowing for in-frame cloning with the α-leader and EC-C1M (EC-CD23 with the first protease cleavage site mutation). The *lz* was created by annealing four oligonucleotides together to give the correct aa sequence. EC-C1M was generated by PCR using C1M in pCDNA-3 as the template along with primers that added a 5′ *Eco*RI site (CTGCTGAATTCTTGGCACTGGGA) and a 3′ *Not*I site (TGATGGATATGCGGCCGCTTCTCA). After restriction enzyme digestion, the three cDNA fragments were cloned together in frame into pCDNA-1-amp (Invitrogen). Sequencing analysis performed by the Nucleic Acid Core Laboratory (VCU) detected several mutations that were corrected using the Quickchange Mutagenesis Kit (Stratagene). Further sequencing analysis confirmed that the final cDNA was correct. Finally, the complete *lz*EC-C1M cDNA was cloned into the high expression vector pCR4 (a generous gift from Dr. Greg Buck, VCU), a plasmid derived from pEF-BOS (21) that was constructed to include additional cloning sites, including *Sal*I and *Cla*I. The cDNA was cloned into pCR4 using PCR to add a 5′ *Cla*I site (TACGACTCAATCGATGGAGACCCAAG CTTG) and a 3′ *Sal*I site (AGGTTCCTTCACGTCGACCCTCTAGCA TT) to the end of the cDNA. Finally, the *lz*EC-C1M cDNA was cloned into pET-5a using a PCR strategy that introduced an *Nhe*I site at the 5′ end (GCTAGCTTGCAAGAACTCCAAGCT) along with the same 3′ oligonucleotide as was used for cloning into pCR4.

Preparation of transient transfected cells

COS cells were collected in log-phase growth, and electroporation was performed as previously described (14). Protein expression was analyzed using ^{35}S metabolic labeling (^{35}S-translabel protein labeling mix (NEN/Dupont, Boston, MA)) of either transiently transfected COS cells, permanently transfected C1M in CHO-K1, or CD23$^+$ in CHO-K1 as previously described (15). Cells were labeled 2 days after transient transfection or 1 day after seeding a medium flask with 5×10^6 cells of permanently transfected CHO cells. Media contained 0.4 mM calcium nitrate, sufficient to allow IgE binding to occur (26). To control for differences in either C1M or native CD23 expression, untransfected CHO cells were added to the CD23$^+$ cells before ^{35}S labeling to give similar total CD23 levels. Supernatants containing ^{35}S-labeled protein were subjected to affinity chromatography as previously described (15). Alternatively, supernatant from transfected cells was isolated, and the concentration of sCD23, EC-CD23, or *lz*EC-C1M was determined using an sCD23 ELISA.

Bacterial expression

EC-CD23 or *lz*EC-C1M in pET-5a was transformed into the *Escherichia coli* strain BL21 using the calcium chloride protocol (27). A 1-liter flask of M9 medium was inoculated with a 5-ml overnight culture of EC-CD23 in pET-5a and incubated, with shaking, until the OD_{600} was 0.6–0.8, at which time the protein was induced by adding 0.4 mM isopropyl thiogalactoside (Sigma, St. Louis, MO), and the bacteria were pelleted after an additional

6–8 h of growth and stored at −70°C. Induction was confirmed by SDS-PAGE analysis of an aliquot of the pellet. The induced protein was found in inclusion bodies and was purified from *E. coli* using a modification of a published procedure (28). Briefly, after thawing the bacteria, the pellet was resuspended in 72 ml of ice-cold solution 1 (10 mM Tris, pH 7.9; 25% sucrose; 100 mM KCl; 2 mM DTT; and 2 mM PMSF), and then 18 ml of ice-cold solution 2 (300 mM Tris, pH 7.9; 100 mM EDTA; and 4 mg/ml lysozyme) was added, and the mixture was chilled on ice for 10 min. Ninety milliliters of ice-cold solution 3 (1 M LiCl, 20 mM EDTA, and 0.5% TX-100) was added, and the material was sonicated (Branson Sonifier 450; VWR, Bridgeport, NJ) at 90% duty cycle, output control 8 (for 1–3 min or until no bacteria particles were present), and placed immediately on ice. The inclusion bodies were pelleted by centrifuging 10 min at 10,000 rpm at 4°C. The pellet was resuspended in 200 ml of ice-cold solution 4 (10 mM Tris, pH 7.9; 0.1 mM EDTA; 0.5 mM LiCl; 0.5% TX-100; 1 mM DTT; and 1 mM PMSF) and then sonicated and pelleted as described above. The pellet was resuspended in 200 ml of ice-cold solution 5 (10 mM Tris, pH 7.9; 0.1 M EDTA; 2% TX-100; 1 mM DTT; and 1 mM PMSF) sonicated and pelleted as described above. The pellet was resuspended in 200 ml of ice-cold solution 6 (10 mM Tris, pH 7.9; 0.1 M EDTA; 0.5% TX-100; 1 mM DTT; and 1 mM PMSF), sonicated, pelleted for the last time, and stored at −20°C. (All chemicals in solutions 1–6 are from Sigma.) The recombinant protein was purified by gel filtration on Sephacryl-200 using 6 M GnCl on a fast protein liquid chromatograph (Pharmacia). The fractions containing the protein were pooled and concentrated to 3–5 ml using an XM10 or YM10 ultrafiltration membrane (Millipore, Bedford, MA) in an Amicon stirred cell protein concentrator (Millipore). The volume was adjusted so that the final concentration of the protein was approximately 10 mg/ml and the protein was stored at 4°C. Protein concentrations were determined using the calculated extinction coefficient of EC-CD23 or *lz*EC-C1M (1.93 and 1.55, respectively). Renaturation of the protein occurred as follows. DTT (10 mM) was added to 1 ml of solution containing approximately 10 mg of protein, and the solution was incubated for 1 h at room temperature. The protein was slowly diluted in 9 ml of refolding buffer 1 (0.5 M Tris, pH 8.6; 6 M GnCl; and 0.1 M oxidized glutathione (Sigma)) and stored at 4°C for 24 h. After 24 h, the 10 ml of protein was slowly diluted, with stirring, into 990 ml of refolding buffer 2 (100 mM Tris, pH 8.6; 1 M GnCl; 1 mM $CaCl_2$; and 3 mM L-cysteine) and stored at 4°C for 36 h. The protein will precipitate if it is added too quickly to either of the refolding buffers. After 36 h, 10 μM *N*α-*p*-tosyl-L-lysine chloromethyl ketone (Sigma) was added, and the protein was concentrated to 2–3 ml using the Amicon protein concentrator. The filtered protein was then dialyzed against the final buffer of choice. The final preparation was analyzed on a 10% SDS-PAGE to check the size, purity, and concentration, and both EC-CD23 and *lz*EC-C1M were essentially homogeneous at this time. Final yields were typically 5–10 mg/ml and 1–2 mg/ml for the EC-CD23 or *lz*EC-C1M, respectively. Different preparations were standardized for use in binding assays by analysis by a sCD23 ELISA (see below).

sCD23 ELISA

The concentration of sCD23, EC-CD23 or *lz*EC-C1M present in supernatant of transfected cells or purified preparations was determined using an assay similar to the IgE ELISA previously described (7), instead using the following Abs. Plates were coated with 20 μg/ml 2G8, and 5 μg/ml Rabbit B was added following incubation with unknown samples. EC-CD23 was prepared in house and used as the standard. A 1:500 dilution of goat anti-rabbit horseradish peroxidase (Southern Biotechnology Associates, Birmingham, AL) was used as the detection Ab followed by the addition of TMB One Step Substrate (Dako, Carpinteria, CA). The reaction was stopped with 0.18 M H_2SO_4 and read at wavelength 450 nm. Standard curves were run and four-parameter analysis was performed using Molecular Devices software. All ELISA determinants were performed with a duplicate series of dilutions. Dilution values that fell in the linear portion of the curve were used for analysis.

Binding analysis

The capacity of membrane-bound chimeric proteins to bind ^{125}I-labeled B3B4 or murine IgE (mIgE) was performed to detect expression of the lectin head and to test for the ability to interact with IgE. Scatchard analysis was performed as previously described (14). Analysis of the CD72-CD23 chimera was performed by adding 2 million cells with 100-fold excess of unlabeled IgE or B3B4 and incubated on ice for 10 min. Subsequently, 10 μg/ml ^{125}I-labeled IgE or 2 μg/ml ^{125}I-labeled B3B4 was added (final volume, 0.5 ml), and the incubation was continued for 60 min at 4°C. Alternatively, chimeric or recombinant proteins were analyzed for the ability to inhibit ^{125}I-labeled mIgE binding to CD23 or FcϵRI (the high affinity receptor for IgE) using CD23$^+$ CHO-K1 cells or C57 mast cells, respectively. The indicated amounts of inhibitor were added to ^{125}I-labeled mIgE and allowed to incubate on ice for 10 min. Two million cells were added, and the incubation was continued for 60 min at 4°C. With all protocols, cell-bound radioactivity was then determined using the phthalate oil cushion procedure (14). Specific binding was determined by subtracting controls that were incubated with 100-fold excess of cold IgE or anti-CD23 (B3B4). Molecules bound per cell were calculated based on the specific activities of the respective proteins.

Results

Replacement of the stalk of CD23 with any stalk other than CD23 abrogates IgE binding

The current model of CD23 indicates that interaction with IgE is in the form of an oligomer (14), indicating that both affinity and avidity play a significant role in this interaction. In an effort to analyze the structural requirements for high affinity/avidity binding to IgE, several different chimeras were made that contained the lectin domain of CD23 (see Fig. 7 for schematic representation of the various domains of CD23) attached to the stalk domain from proteins known to form oligomers. A soluble chimera was made that contained the lectin domain of CD23 and the stalk from MBP-C. MBP-C contains a collagen-like stalk that is known to mediate trimer as well as higher oligomer formation (20). To determine both whether MBP-CD23 was being expressed as a monomer or an oligomer and whether the protein could bind IgE, COS cells were transiently transfected with MBP-CD23 and labeled with ^{35}S-translabel, and affinity chromatography was used to purify the labeled chimera. As a positive control for protein expression and IgE binding, CHO-K1 cells transfected with EC-CD23 were used. Fig. 1 shows the labeled protein analyzed by SDS-PAGE run in reduced (Fig. 1*A*) or nonreduced (Fig. 1*B*) sample buffer. In both gels, it is evident that the MBP-CD23 cannot bind IgE (Fig. 1, *lane 4*), even though it is recognized by B3B4 (Fig. 1, *lane 3*); neither protein bound to a nonspecific affinity column (data not shown). This lack of affinity for IgE is not due to lack of oligomerization, as shown by the presence of higher m.w. forms of MBP-CD23 (Fig. 1, *lane 2*) isolated in nonreducing sample buffer. Note that the stalk of MBP is capable of forming oligomers even in the presence of SDS. These data indicate that while the MBP stalk does cause oligomer formation, oligomerization of the lectin head alone is not sufficient to allow for recognition of IgE, at least not by utilizing a collagen-like stalk.

Two membrane-bound chimeras were made that contained the stalk of CD72. CD72 is a type II integral membrane protein and a member of the C-type animal lectin family (29). Sequence analysis and homology pattern search indicate that the stalks of both CD23 and CD72 contain the same heptad periodicity indicative of an α-helical coiled-coil structure (30, 31). Thus, a membrane-bound chimera was made containing the cytoplasmic, transmembrane, and stalk domains of CD72 and the lectin head of CD23. Both transient (data not shown) and stable transfections determined that CD72-CD23 could not bind mIgE with a measurable affinity, even though the epitope recognized by B3B4 was present (Fig. 2). The saturation analysis used is the same as used previously (14) and detects both high and low affinity binding to CD23 (Figs. 3 and 4). Since CD72-CD23 was unable to bind mIgE even though the stalk of CD72 is highly homologous to CD23, it was argued that not enough of the lectin domain of CD23 had been included to allow for both high and low affinity binding. Surfactant-D, a member of the collectin family, has been shown to contain a 35-aa neck domain that mediates the formation of an α-helical coiled-coil region (32). Studies indicate that the neck and lectin domain of surfactant-D alone not only were capable of trimerizing, but also retained the ability to bind the surfactant-D ligand in a multivalent manner

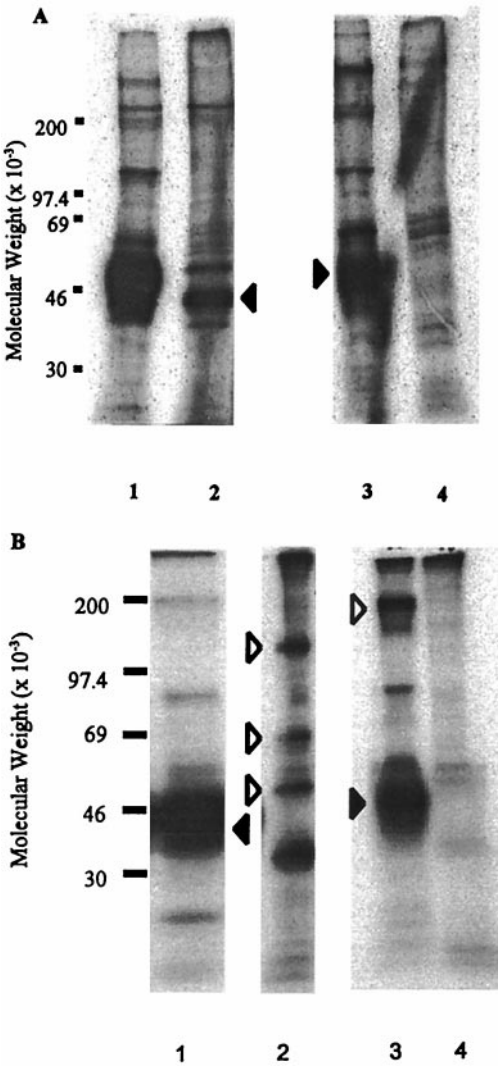

FIGURE 1. MBP-CD23 is expressed as an oligomer, but it cannot bind mIgE. The MBP-CD23 chimera was expressed in COS cells in a transient transfection and labeled overnight with ^{35}S-translabel as described in *Materials and Methods*. EC-CD23-transfected cells were labeled and used as a positive control. Supernatants were subjected to affinity chromatography and were isolated by boiling in reduced (*A*) or nonreduced (*B*) SDS sample buffer. Samples were run on an 8–18% gradient gel. Both EC-CD23 (*lane 1*) and MBP-CD23 (*lane 2*) are recognized by B3B4. EC-CD23 (*lane 3*) but not MBP-CD23 (*lane 4*) can bind mIgE. Samples analyzed in nonreduced sample buffer (*B*) indicate that both EC-CD23 (*lane 1*) and MBP-CD23 (*lane 2*) form oligomers, but this does not affect the ability of MBP-CD23 to bind mIgE (*lane 4*). Arrowheads indicate the monomeric form of MBP-CD23 or EC-CD23 (solid arrow) and oligomeric forms (open arrow).

(32). Since both CD72 and CD23 are members of the same family as surfactant-D, and the carboxyl portion of the stalk of CD23 contains aa that are conducive to forming a neck region, it was thought that a neck domain may be responsible for orienting the lectin heads such that it could properly interact with IgE. To test this, a second chimera was made that was similar to CD72-CD23, but in addition contained the neck domain of CD23 (see Fig. 7). The CD72-CD23neck chimera contained the same domain from CD72 attached to the neck and lectin domain of CD23. The putative neck domain of CD23 contained aa 157–188. In contrast to the reports on surfactant-D (32), the addition of the neck had no effect on mediating an interaction with mIgE. Expression analysis of the

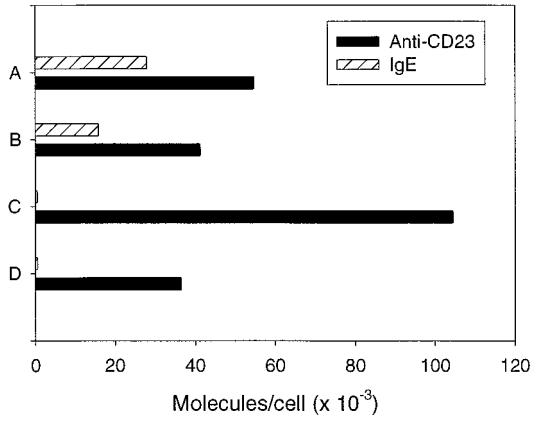

FIGURE 2. CD72-CD23 can bind B3B4 but cannot bind mIgE. The CD72-CD23 chimera was expressed in COS cells in a stable transfection using CHO/dihydrofolate reductase$^-$ cells and binding analysis was performed as described in *Materials and Methods* to ascertain the affinity of the chimera for mIgE. CD23$^+$ CHO-K1 (*A*) or CD23$^+$ COS (*B*) cells were used as positive controls, and untransfected cells were used as the negative control to determine nonspecific binding. The graphs shows that while the B3B4 epitope of CD23 is expressed in two separate clones (clone 10 (*C*) and clone 3 (*D*)), the chimera cannot bind mIgE. Note that the IgE binding to CD23 is the expected approximate binding (50%) as seen with anti-CD23 at the saturating concentrations of ligand (6).

chimera using a transient transfection in COS cells or a stable expression in CHO-K1 determined that the chimera could not bind IgE (data not shown), even though the B3B4 epitope was present. These data indicate that the absence or presence of the neck domain used with the homologous stalk of CD72 is not sufficient to allow for the lectin domain of CD23 to bind IgE.

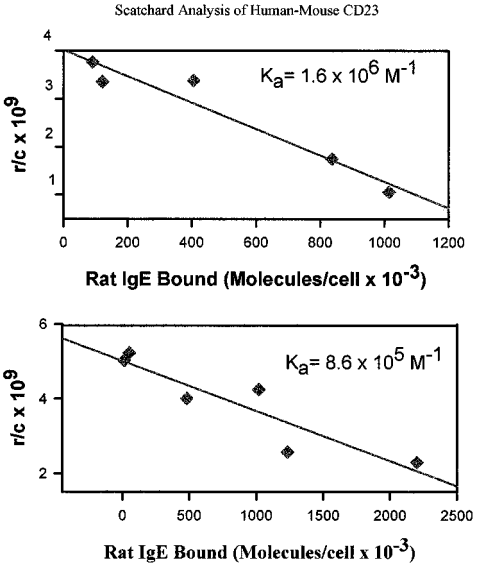

FIGURE 3. Human-mouse CD23 can only bind rIgE with a low affinity. The human-mouse CD23 chimera in pEF-BOS was expressed in COS cells in a transient transfection, and binding analysis was performed as described in *Materials and Methods* to ascertain the affinity of the chimera for rIgE. The data were analyzed by Scatchard analysis, and graphs (representative of two separate experiments) show that the chimera can only bind rIgE with a single low affinity; the association constants for low affinity binding are shown (*top right*).

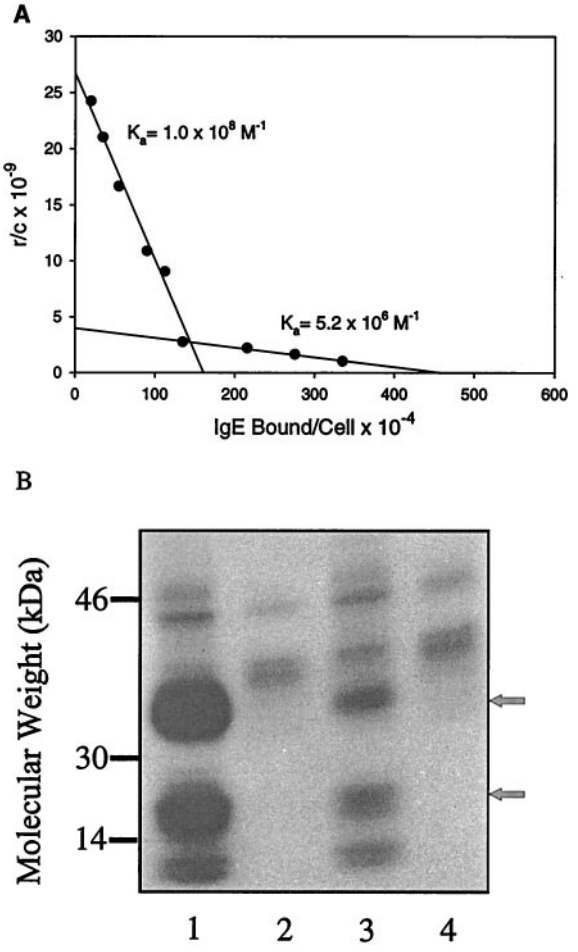

FIGURE 4. C1M binds IgE normally but shows a reduced amount of cleavage compared with CD23. *A*, Saturation analysis of ^{125}I-labeled IgE binding to C1M-transfected CHO was performed as described in *Materials and Methods*. Data were plotted so as to give a Scatchard plot, and the dual affinity seen is quite similar to that previously reported for wild-type membrane CD23 (14). *B*, CD23- or C1M-transfected CHO-K1 cells were labeled with ^{35}S-translabel, and supernatants were subjected to affinity chromatography as described in *Materials and Methods*. Bound protein was boiled in reduced sample buffer to remove the protein from the affinity matrix and analyzed by SDS-PAGE. Neither CD23 (*lane 2*) nor C1M (*lane 4*) bound to a nonspecific affinity matrix (B1E3). Both CD23 (*lane 1*) and C1M (*lane 3*) bound the anti-CD23 (B3B4) Affi-Gel, but note that the amount of material (sCD23) released into the supernatant is substantially less (position is indicated by the arrows (right), which indicate 38- and 28-kDa forms of sCD23, respectively) in the supernatant isolated from C1M-transfected cells compared with CD23-transfected cells.

Analysis of human-mouse CD23 chimera

The data from the above chimeras indicated that the stalk of CD23 may not only be important for oligomerization, but may also be important in regulating the interaction with IgE. A human-mouse CD23 chimera was previously made by this laboratory (14) consisting of the lectin domain of murine CD23 linked to the human stalk plus transmenbrane and cytoplasmic domains. This chimera was found to bind mIgE (14) with a dual affinity. Murine CD23, as expressed on the cell surface, can interact with both mIgE and rIgE (23). Scatchard analysis was performed to investigate whether this chimera could also bind rIgE. The data from initial experiments indicated that human-mouse CD23 could not bind rIgE. To determine whether low affinity binding was occurring, the cDNA for the chimera was moved into the high expression vector pEF-BOS. The results from the Scatchard analysis determined that while the chimera could bind mIgE with both a high and low affinity (14), it could only bind rIgE with a low affinity (see Fig. 3). These data indicate that while the human stalk of CD23 does mediate oligomerization as determined by the ability of the chimera to bind mouse IgE with a dual affinity, it does not enable the chimera to interact with rIgE, indicating that there may be another site(s) present that is responsible for this interaction.

C1M is not as sensitive to proteolytic degradation

Work on EC-CD23 combined with the information gained from the above-mentioned chimeras suggested that for high affinity binding to IgE to occur, the entire stalk of CD23 must be present. At the same time, the stalk of CD23 is extremely susceptible to proteolytic degradation from the cell surface (33). In an attempt to create a more stable form of CD23, a cleavage-resistant mutant (C1M) was created by mutating the first cleavage site from an asparagine to an alanine in the hope that this mutation would render the stalk more resistant to proteolytic degradation. The C1M mutation had no effect on binding of IgE, as shown by the Scatchard analysis in Fig. 4*A*, in which dual affinity binding is shown. This is analogous to binding by wild-type CD23 (14). Release of sCD23 from C1M-transfected cells was analyzed by ^{35}S metabolically labeling CD23- or C1M-transfected CHO-K1 cells, subjecting supernatants to affinity chromatography and analyzing by SDS-PAGE. Note that this analysis assumes the same specific activity for C1M and CD23 from their respective transfected cell populations. To control for this as much as possible, the total level of receptor expression was first determined by B3B4 binding analysis (data not shown). Since the wild-type CD23 was expressed at a higher level, untransfected CHO cells were added to the wild-type CD23-expressing CHO cells before ^{35}S labeling. This gave a similar CD23 level in both populations as judged by B3B4 binding analysis. The experiment shown in Fig. 4*B* is representative of three of similar design. In all cases, there was a drastic reduction in the release of sCD23 from C1M-transfected cells (Fig. 4*B*, *lane 3*) compared with CD23-transfected cells (Fig. 4*B*, *lane 1*). These data indicate that mutating the first cleavage site does result in a more stable protein.

Addition of a stabilizing motif to the amino terminus of CD23 results in high affinity/avidity binding to mIgE

The creation of C1M resulted in the formation of a more stable form of CD23. This mutation was combined with the addition of a protein motif to the amino terminus of EC-CD23 that would allow for more stable trimer formation than normally seen with EC-CD23. Fanslow et al. (25) reported the use of a modified isoleucine zipper for the preparation of a soluble trimeric CD40L. The use of this motif is based on x-ray crystallography, which demonstrated that replacing leucines with isoleucines results in the creation of stable trimers (34). Thus, this approach was extended to EC-CD23. The modified isoleucine zipper was fused to the amino terminus of EC-CD23 (including the C1M mutation) to force the protein to form a stable trimer (see Fig. 7 for schematic representation of *lz*EC-C1M). The cDNA of EC-C1M was cloned in frame to the isoleucine zipper, resulting in the creation of *lz*EC-C1M. Optimal expression of the chimera was obtained using *lz*EC-C1M cloned into the high expression vector pCr4. COS cells transiently transfected with *lz*EC-C1M or EC-CD23 were metabolically labeled with ^{35}S-translabel. The supernatants were subjected to affinity chromatography and analyzed by SDS-PAGE. Fig. 5 shows the data from these experiments, which indicate that the *lz*EC-C1M can bind mIgE (Fig. 5, *lane 6*) just as well as it can bind B3B4 (Fig. 5, *lane 5*). Fig. 5 (*lane 7*) also shows that the isoleucine

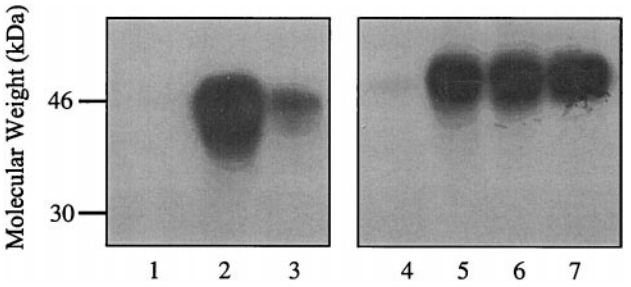

FIGURE 5. *lz*EC-C1M can bind mIgE with a higher affinity/avidity than EC-CD23. EC-CD23- or *lz*EC-C1M-transfected COS cells were labeled with ^{35}S-translabel, and supernatants were subjected to affinity chromatography as described in *Materials and Methods*. Bound protein was eluted from the affinity matrix by boiling in reduced sample buffer and analyzed by SDS-PAGE. Neither EC-CD23 (*lane 1*) nor *lz*EC-C1M (*lane 4*) bound to a nonspecific affinity matrix (B1E3). Both EC-CD23 (*lane 2*) and *lz*EC-C1M (*lane 5*) bound the anti-CD23 (B3B4) Affi-Gel, but the *lz*EC-C1M interacted with the mIgE (*lane 6*) Affi-Gel with a much higher affinity than the EC-CD23 (*lane 3*). The leucine zipper portion of the protein was expressed in the chimera as the addition of M15, an anti-leucine zipper Ab, to the supernatant from *lz*EC-C1M-transfected COS cells, which allowed for the chimera to be immunoprecipitated by a protein A/protein G Affi-Gel (*lane 7*).

zipper motif is expressed, since the protein can be immunoprecipitated with a protein A/protein G affinity matrix that has been incubated with an anti-leucine zipper Ab, M15, and supernatants from the *lz*EC-C1M-transfected cells. The *lz*EC-C1M is also capable of binding mIgE better than EC-CD23, as can be seen by comparing Fig. 5, *lane 6*, the *lz*EC-C1M with Fig. 5, *lane 3*, EC-CD23. Data are representative of two experiments. Thus, while the actual affinity/avidity remains to be determined, the addition of the *lz* motif clearly results in increased binding capacity to IgE.

l*zEC-C1M can inhibit binding of IgE to both CD23 and Fc*ϵ*RI*

The relative affinity of the *lz*EC-C1M for IgE appears to be much higher than that of EC-CD23, and this result is most likely due to the stable trimeric conformation of the chimera. Because of the increased ability to interact with IgE, the *lz*EC-C1M was analyzed for its ability to inhibit mIgE binding to CD23 or FcϵRI. Binding analysis was performed using CD23$^+$ CHO-K1 or C57 mast cells, adding a constant concentration of ^{125}I-labeled mIgE and an increasing concentration of the *lz*EC-C1M. Percentage inhibition was determined by using the binding of ^{125}I-labeled mIgE to the cells that were incubated with media from mock-transfected COS cells. Fig. 6*A* shows that *lz*EC-C1M can inhibit the binding of mIgE to CD23$^+$ CHO-K1 cells in a dose-dependent manner, and this inhibition is similar regardless of whether the source of *lz*EC-C1M is *E. coli* or COS cell supernatants, indicating that the *lz*EC-C1M can be successfully refolded from bacterial inclusion bodies. Interestingly, we were unable to achieve more than ~50% inhibition of binding to the CD23$^+$-CHO cells (see *Discussion*). The *lz*EC-C1M was a more effective inhibitor of IgE binding to the FcϵRI again, the bacterium-produced *lz*EC-C1M works essentially the same as *lz*EC-C1M in COS cell supernatants. Assuming a trimeric configuration, the m.w. of the *lz*EC-C1M is quite similar to that of IgE. Thus, an approximately equimolar concentration of *lz*EC-C1M and ^{125}I-labeled IgE (100 ng) gives ~70% inhibition of binding to the FcϵRI. Shown also is the essential lack of inhibition of binding using a similar dose range of EC-CD23, both with respect to CD23$^+$CHO or C57 mast cells. In a separate experiment, we also examined the capacity of soluble CD40 ligand trimer to inhibit IgE binding to C57 or CD23$^+$ cells. CD40 ligand trimer

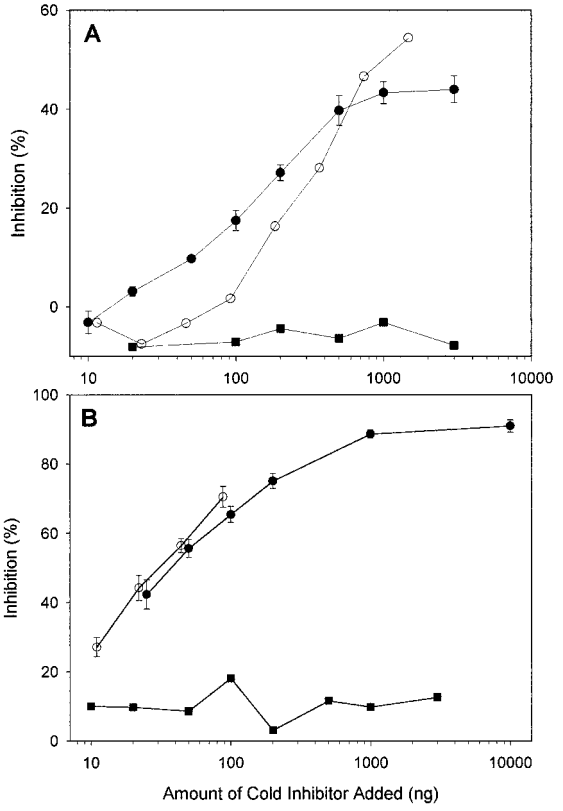

FIGURE 6. Capacity of EC-CD23 vs *lz*EC-C1M to inhibit ^{125}I-labeled mIgE binding to CD23$^+$ CHO-K1 cells and C57 mast cells. Increasing volumes of supernatant from *lz*EC-C1M-transfected COS cells (●) or purified *lz*EC-C1M (○) or EC-CD23 (■) prepared in *E. coli* were added to CD23$^+$ CHO-K1 cells (*A*) or C57 mast cells (*B*) in a binding assay as explained in *Materials and Methods*. Percentage inhibition was calculated as follows: (cpm experimental − cpm 100-fold excess of unlabeled IgE)/(cpm control with no inhibitor − cpm 100-fold excess of unlabeled IgE) × 100. Average inhibition from three experiments is shown ± 1 SE, except for COS cell supernatant inhibition of CD23$^+$-CHO, which is a single experiment.

contains the same modified *lz* trimer at its amino terminus; again, the purpose is to mediate trimerization of the molecule (18). No inhibition of IgE binding was seen (data not seen), ruling out a trivial explanation for the data, namely that the *lz* sequence influences IgE binding to CD23 or the FcϵRI. To ensure that the mast cell line used was not aberrantly expressing the low affinity receptor for IgE, the cells were analyzed by FACscan for their ability to bind mIgE as well as looking at CD23 expression. While the cells could bind mIgE, there was no CD23 expressed on these mast cells (data not shown).

Discussion

Previous work on CD23 from several different laboratories has determined that CD23 must be an oligomer to bind IgE with a high affinity (14, 30, 35); this includes early data from this laboratory, which indicated that CD23 can self-associate to form a trimer or tetramer as indicated by its m.w. following treatment with chemical cross-linking reagents (36) and that the stalk region of CD23 (aa 55–157) is important in this oligomerization. Several chimeric proteins were previously useful in investigating various aspects of CD23 structure function. These data are summarized in Dierks et al. (14) and Bartlett et al. (15) and allowed the conclusion that the cytoplasmic and transmembrane domains of CD23 have no role in

IgE binding and function only to anchor the protein on the cell surface. On the other hand, the stalk of CD23 is required to bind IgE, either because it is needed to mediate oligomerization or because it can directly interact with IgE. This study extended this aspect of the stalk requirement through the analysis of several additional unique CD23 chimeras, as well as further analysis of the human-mouse CD23 chimera.

The latter human-mouse chimera was previously reported to interact with IgE with dual affinity (14). Studies described here explored this further by looking at the ability of human-mouse CD23 to bind rIgE (Fig. 3). Using a high protein expression system, the human-mouse CD23 could not bind rIgE even though murine CD23 normally interacts with both rat and mouse IgE (23). This indicates that there is one or more site within the stalk that bind IgE and there is enough homology between the stalk of human and mouse CD23 that binding to mIgE was unaffected, but the homology between the region required to bind rIgE was not as conserved. While the determination of these exact sites of interaction will require further study, this disparity points to the fact that the role of the stalk may not be as simple as to only mediate oligomerization.

Several new chimeras were made in the attempt to create a stable oligomeric form of CD23 that could bind IgE with an affinity higher than that of EC-CD23. The initial chimeras utilized the lectin head of CD23 combined with stalks from proteins known to form oligomers. Interestingly, neither the stalk from MBP, a protein known to form trimers and hexamers via its collagen-like stalk (20), nor the stalk of CD72, a protein homologous to CD23 with respect to the predicted coiled-coil structure of the stalk, enabled the respective chimeras (Figs. 1 and 2) to bind IgE, analogous to the results seen with the Ly-49-CD23 chimera composed of the stalk of Ly-49 (15). Also, the addition of the neck domain, by analogy with surfactant-D (32), did not improve the binding capacity of the CD72-CD23 chimera. In studies performed with only the neck and lectin domain of surfactant-D, surfactant-D was not only shown to be capable of trimerizing, but it also retained that ability to bind its ligand in a multivalent manner (32). Since both CD72 and CD23 are members of the same family as surfactant-D, and the carboxyl portion of the stalk of CD23 contains aa that are conducive to forming a neck region, it was thought that a neck domain may be responsible for orienting the lectin head such that it could properly interact with mIgE. In all cases, conformation of in-frame chimeric production was monitored via the interaction with anti-CD23. These data combined with data from the previously mentioned chimeras imply that replacing the stalk of CD23 with any other stalk completely abrogates the capacity to bind IgE.

EC-CD23 was previously created to investigate the role that the stalk plays in mediating a high affinity/avidity interaction with IgE (15). A problem found with the full-sized extracellular EC-CD23 was the stability of the molecule. The creation of sCD23 naturally occurs by proteolytic cleavage within the stalk, resulting in the release of a 38-kDa fragment, with subsequent cleavage forming 35-, 28-, and 25-kDa species (6, 15, 37). Murine sCD23 can inhibit binding of IgE to $CD23^+$ cells at high concentrations (15), but it cannot bind IgE with an affinity/avidity comparable with that seen with the membrane form. The C1M mutant was made in an attempt to create a form of CD23 that was not subject to this same degree of proteolytic degradation. The mutation of the first cleavage site from an asparagine to an alanine did result in a form of CD23 that released decreased quantities of sCD23 (see Fig. 4*B*). While these data assume similar specific activities for the ^{35}S labeling, adjustment of CD23 levels at initiation of labeling, combined with the strong decrease in sCD23 seen, indicate that this mutation had the desired effect. This mutation was utilized in the creation of a soluble chimeric CD23 that would allow further investigation of the role that a stable stalk plays in mediating the interaction with IgE.

The chimeric data combined with the data from EC-CD23 demonstrated that the stalk of CD23 was more important in promoting both trimerization and IgE binding than was previously thought. Previous work with EC-CD23 determined that the addition of the entire stalk did allow for a higher affinity/avidity with IgE compared with sCD23; however, the affinity is still a log lower than the affinity seen with CD23 (15). The most feasible explanation for this result is that the trimeric form of EC-CD23 was not sufficiently stabilized, resulting in disassociation of the trimer to that of a monomer. If a protein motif could be added to the amino-terminal portion of EC-CD23, stabilizing the trimer conformation in the same manner that the membrane stabilizes CD23 on the cell surface, it was anticipated that such a recombinant protein would bind IgE with an affinity comparable with that of membrane CD23. The *lz*EC-C1M made to test this hypothesis contains a modified isoleucine motif that has been shown to form stable trimers (34). As mentioned earlier, the stalk contained a mutation to the first cleavage site, resulting in a protein that was not subject to the same degree of proteolytic degradation normally seen with CD23. The resulting capacity of the chimera to bind IgE was clearly superior to that of EC-CD23 (Fig. 5). While actual affinity/avidity measurements have yet to be performed, the increased binding suggests that the capacity to interact with IgE is at least as effective as native membrane CD23. Thus, these data indicate that the inability of sCD23 to bind IgE with a significant affinity/avidity results from its inability to form a stable trimer due to the lack of the amino portion of the stalk combined with the loss of the membrane to provide a surface to force oligomerization.

The increased capacity to interact with IgE is dramatically emphasized in that the *lz*EC-C1M exhibited an excellent capability to block IgE binding to the FcϵRI. In early work on sCD23, Suemura et al. (38) found that a 10,000- to 100,000-fold excess of sCD23 was required to inhibit IgE binding to the FcϵRI. The addition of similar concentrations of EC-CD23 expressed in COS cells resulted in a minimal inhibition of IgE binding to FcϵRI and to $CD23^+$ cells. The results with *lz*EC-C1M indicate that when high affinity/avidity binding to IgE is present, only slight excesses of this sCD23 chimera would be necessary to effectively block the interaction between IgE and FcϵRI. It is noted that the inhibition does not reach 100%, even at the highest dose of *lz*EC-C1M tested. The reason for this is unclear and will require further investigation using *lz*EC-C1M from both prokaryotic and eukaryotic sources. Such studies are underway.

The ability of *lz*EC-C1M to inhibit mIgE binding to FcϵRI argues that what has been termed "affinity" should really be termed "avidity," since the stabilization of the extracellular domain of CD23 allowed for a stronger interaction with IgE. The affinity between two proteins is determined by the strength of binding, while the avidity is dependent on a combination of binding sites (39). In other words, both EC-CD23 and *lz*EC-C1M may have the same affinity, but the presence of the isoleucine zipper results in the creation of a stable trimer allowing the avidity between IgE and the *lz*EC-C1M to be higher than that of EC-CD23 because more than one lectin head would be capable of binding to IgE. Fig. 7 shows a schematic representation of how the leucine zipper stabilized EC-C1M by forcing it to form a stable trimeric structure.

The ability of *lz*EC-C1M to form higher oligomers combined with the fact that CD23 is multivalent may also explain why there is only a maximum of ~50% inhibition of mIgE binding to $CD23^+$ cells. Lee and Conrad (40) demonstrated the multivalency of CD23 in studies showing the interaction of more than one IgE molecule by a single CD23 molecule. Therefore, any inhibition

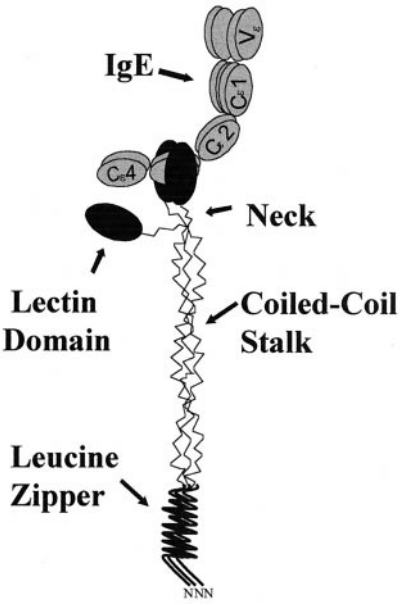

FIGURE 7. Schematic representation of *l*zEC-C1M. The *l*zEC-C1M contains a modified isoleucine zipper motif attached to the amino terminus of the entire extracellular domain of CD23. The lectin head of CD23 consists of aa 189 to the end; the putative neck domain consists of aa 157–188, and the stalk domain contains aa 55–157.

mediated by EC-CD23 would be masked by the ability of *l*zEC-C1M to oligomerize with CD23 expressed on the cell surface. If this was occurring, mIgE would be retained on the cell surface even though it was bound to the *l*zEC-C1M. This will be directly tested once larger quantities of *l*zEC-C1M are available.

The implications for this protein are important with respect to a potential role as a therapeutic agent for use with IgE-mediated disease. Therapies directed against preventing allergy either by inhibiting the protease responsible for cleaving CD23 (41) or through the use of a humanized anti-IgE (42–44) are presently under investigation. Both in vitro data using cells overexpressing CD23 (7) and in vivo data from CD23-transgenic mice (7, 8) corroborates that CD23 can be involved in IgE down-regulation. The *l*zEC-C1M represents an additional alternative for blocking the interaction of IgE with the Fc receptors expressed on mast cells and basophils. Studies in progress will investigate whether this strategy offers advantages over the anti-IgE and protease inhibition strategies mentioned above.

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