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Expression of Novel Secreted Isoforms of Human Immunoglobulin E Proteins*

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Four human IgE isoforms produced by alternative splicing of the epsilon primary transcript were expressed as chimeric mouse/human anti 5-dimethylamino-1-naphthalenesulfonyl antibodies in the murine myeloma cell line Sp2/0. The four isoforms include the classic secreted form and three novel isoforms with altered carboxyl termini. All of these isoforms lack the transmembrane region encoded by the M1/M1′ exon and are therefore predicted to be secreted proteins. When expressed in Sp2/0 cells, three of the IgE isoforms are assembled into complete molecules of two Ig heavy chains and two Ig light chains, whereas the fourth isoform is predominately assembled into half-molecules of one Ig heavy chain and one Ig light chain. All four isoforms are secreted with similar kinetics. In contrast, when the isoform containing the Cϵ4 domain joined directly to the M2′ exon (IgE grandé) is expressed in the J558L cell line, it is degraded intracellularly, suggesting a cell line-dependent regulation of secretion. These data show that these novel isoforms of human IgE, predicted to occur from *in vivo* and *in vitro* mRNA analysis, can be produced and secreted by mammalian cells. The different forms of IgE may have physiologically relevant but distinct roles in human IgE-mediated immune inflammation. The availability of purified recombinant human IgE isoforms makes it possible to analyze the functional differences among them.

Alternative RNA splicing determines the production of secreted *versus* membrane-bound forms of immunoglobulins (1, 2). This is accomplished in mammals by the alternative usage of either a secreted terminus at the end of the last constant region domain or two downstream exons (M1 and M2) that encode the transmembrane and intracellular amino acids. Splicing to the M exons removes from the transcript the nucleotides that encode the hydrophilic COOH terminus and polyadenylation signal for the smaller, secreted form of the Ig.

The one functional genomic locus encoding human epsilon heavy chain contains four Ig domain exons (Cϵ1 to Cϵ4) and the two membrane exons (M1 and M2). We (3–5) and others (6, 7) have previously shown that RNA prepared from the IgE-producing human cell line AF-10 and from fresh B lymphocytes stimulated to make IgE contain a variety of epsilon mRNAs produced by alternative splicing. In contrast to what is observed with other isotypes, the most common form of mRNA encoding membrane IgE is produced by splicing to a novel splice acceptor 156 base pairs upstream of the normal M1 acceptor site (4, 7). The M1′ exon produced using this splice acceptor encodes 52 novel amino acids that are largely hydrophilic followed by the amino acids normally encoded by M1.

Other alternatively spliced epsilon mRNAs are present that encode a series of potentially secreted proteins. The splicing events that generate these mRNAs utilize several novel exons including M2′, M2″, and Cϵ5 in addition to the classic secreted form (see Fig. 1*A*). The M2′ exon is created by splicing directly from Cϵ4 to the normal M2 splice acceptor. The omission of M1 results in a frameshift in M2, which creates an open reading frame encoding 136 hydrophilic amino acids (*i.e.* M2′). M2″ is a short tail (8 amino acids) created by splicing from Cϵ4 to a splice acceptor located within the M2′ exon. The reading frame of M2″ is different from that of M2′ (4, 5).

It is of great interest whether these novel mRNAs encode functional proteins and whether these various forms of IgE play distinct roles in the immune response. Using polyclonal anti-peptide antibodies, we have detected the protein product of one of these novel splice variants in the supernatant and cytoplasm of AF-10 cells and in serum from a patient with IgE myeloma (3). However, the low level of IgE present in normal serum makes it impossible to isolate sufficient quantities with adequate purity for definitive functional studies. Human IgE is the least abundant Ig with average serum concentrations (125 ng/ml) generally 100,000-fold less than IgG in normal individuals. Purification from serum would be further confounded by the similar molecular size of several of the splice variants. Therefore, we have focused on developing expression systems for the production of each epsilon splice variant.

In a recent study, Batista *et al.* (8) report the expression in J558L murine myeloma cells of five constructs that encode individual splice variants of IgE (8). The conclusions of this study were that only one form of IgE is detectable on the surface of the transfected cells and only one form of soluble IgE is secreted by myeloma cells. These forms were found to correspond to the CH4-M1′ membrane-bound and the classic secreted (CH4-S) forms of IgE, respectively. Although Western blotting of protein secreted by the cell line U266 revealed heterogeneity of epsilon chains, Batista *et al.* (8) conclude that this is the result of differential glycosylation. They further conclude that the novel epsilon isoforms produced by alternatively spliced mRNAs are degraded intracellularly

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and therefore cannot constitute functionally relevant forms of IgE.

In the present study, we demonstrate the expression and secretion of four soluble isoforms of human IgE by the murine myeloma cell line Sp2/0. The isoforms examined are classic secreted (CH4-S), CH4-M2′, CH4-M2″, and CH4′-CH5. We designate these proteins as IgE classic, IgE grandé, IgE tailpiece (IgEtp), and IgE chimeric CH4 (IgEψCH4), respectively. All four isoforms show similar kinetics and efficiency of assembly and secretion, although one of the isoforms (*i.e.* IgEψCH4) is secreted predominately as HL[1] half-molecules. Furthermore, the production of secreted protein is shown to depend on the murine myeloma cell line used for expression.

EXPERIMENTAL PROCEDURES

Vectors and Proteins—To create isoform-specific epsilon constructs, a portion of the human epsilon gene encoding Cϵ1 through Cϵ4 was fused in-frame to three RT-PCR clones. The RT-PCR clones span the downstream sequences resulting from alternative splicing events as well as a portion of Cϵ4 (see Fig. 1*B*). RT-PCR cloning of 3′ portions of the various epsilon mRNAs has been reported (4, 5). Briefly, total RNA was isolated from both the IgE-producing human myeloma AF-10 and from purified B cells stimulated to produce IgE with interleukin-4 and CD-40 monoclonal antibody. The RNA was then reverse-transcribed using oligo$(dT)_{15}$ primer (Boehringer Mannheim) and mouse Moloney leukemia virus reverse transcriptase (Life Technologies, Inc.). cDNA was then used as substrate for PCR reactions using upstream primers located within Cϵ4 at Asp^{497}–Gln^{504} (IgEψCH4) or at Glu^{541}–Val^{549} (IgE grandé and IgEtp) (5) and a downstream primer located 21–42 nucleotides 3′ of the M2′ exon (4). PCR reaction mixtures contained 10% Me_2SO, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM $MgCl_2$, and 100 μg/ml nuclease-free bovine serum albumin. Primer concentration was 0.5 μM. Reactions were carried out for 40 cycles at the following temperatures: melting for 1 min at 94 °C, annealing for 1 min at 72 °C, and extension for 1 min at 72 °C.

Isoform-specific epsilon chain genes were created by first using PCR mutagenesis to create novel *Xba*I or *Nhe*I restriction sites within the Cϵ4 coding region of each of these RT-PCR clones and an *Eco*RI site at the 3′ end of each clone (before the polyadenylation addition signal). PCR mutagenesis was then used to create the identical mutations within the Cϵ4 exon of the human genomic epsilon gene. The *Xba*I site was created at arginine 520; the *Nhe*I site was added at alanine 543. In all cases introduction of the restriction sites did not alter the amino acid sequence. The *Xba*I or *Nhe*I sites were then used to fuse the various downstream sequences with the epsilon gene. The 3′-untranslated region (UTR) from human γ3 heavy chain gene had been provided with an *Eco*RI site 5′ of the polyadenylation addition signal (9) and was substituted for the 3′-UTR of the human epsilon gene by ligation at the *Eco*RI sites created at the 3′ ends of the RT-PCR clones. The genes encoding the different epsilon isoforms were then cloned into pSV2 gpt containing the coding sequence for a heavy chain variable domain specific for the hapten dansyl chloride under control of the Ig heavy chain promoter and Ig heavy chain enhancer (9). This was done by ligation at the *Bam*HI site at the 3′ end of the 3′-UTR (see Fig. 1*C*) and by ligation of the *Xho*I site immediately 5′ of the Cϵ1 exon with a *Sal*I site 3′ of the Ig heavy chain enhancer in the expression vector. The *Sal*I and *Xho*I sites were destroyed in the ligation and are indicated by Δ in Fig. 1*C*. The anti-dansyl light chain used is a chimeric kappa chain consisting of a murine variable (V_L) domain and human C_κ domain. (10).

Cells—Sp2/0 and J558L murine myeloma cells and various transfectants thereof were carried in Iscove's modified Dulbecco's medium (Irvine Scientific, Santa Ana, CA) containing 5% bovine calf serum (Hyclone, Logan, UT). A stable anti-dansyl light chain-producing line (TWS) was established from Sp2/0 cells as described previously (9). Stable IgE-producing cell lines were created by electroporation of the isoform-specific heavy chain in the pSV2 gpt expression vector into TWS (9). Briefly, 10 μg of DNA linearized at the *Bam*HI site was added to 10^7 TWS cells in 0.9 ml phosphate-buffered saline, and the cells were pulsed at 200 V with 960 microfarads in a 0.4-cm electrode gap cuvette (Bio-Rad). Stable transfectants were selected for growth in medium containing 42 μg/ml hypoxanthine, 1 μg/ml xanthine, and 2.5 μg/ml mycophenolic acid. Clones producing IgE were identified by ELISA using plates coated with dansyl chloride-bovine serum albumin. Supernatants from clones were added to wells, and bound protein was detected using goat anti-human kappa chain conjugated to alkaline phosphatase (Sigma). Nonsecreting transfectants of J558L were identified by lysis of $\sim 5 \times 10^5$ cells in lysis buffer (lysis buffer = 0.5% Nonidet P-40, 50 mM Tris, pH 7.0, 150 mM NaCl, and 5 mM EDTA) and analysis of lysates by ELISA using plates coated with the anti-human IgE monoclonal antibody CIA 7.12 (see ELISA section below). Bound protein was detected using goat anti-human epsilon chain conjugated to alkaline phosphatase (Sigma).

Isoform-specific ELISA—The anti-IgE monoclonal antibody CIA-7.12 that recognizes an epitope at the Cϵ2/Cϵ3 boundary was used as the coating reagent as described (11). After blocking, the cell culture supernatants containing expressed IgE isoforms were incubated for 2 h at room temperature. CIA-7.12 or a 1:500 dilution of α-2331, a rabbit antiserum generated against a peptide corresponding to the COOH-terminal 10 amino acids of IgE grandé (3) was then added to the microplate and incubated for 2 h at room temperature followed by a 2-h incubation with goat anti IgG conjugated to alkaline phosphatase (Sigma).

Western Blotting—Western blotting analysis had been previously described (3–5). Briefly, the expressed IgE isoforms were immunoprecipitated from the cell culture supernatants with monoclonal antibody CIA-7.12 coupled to Sepharose 4B (Pharmacia Biotech Inc.). After transfer, the nylon membrane was blocked by 4% bovine serum albumin/phosphate-buffered saline/Tween for 4 h at room temperature. For ϵ protein detection, the blot was probed with goat anti-human IgE (ϵ chain-specific) conjugated to alkaline phosphatase (Kirkagaard and Perry Laboratories, Inc., Gaithersburg, MD). For detection of IgE grandé, the blot was incubated with 1:200 diluted α-2331 overnight at 4 °C followed by a 2-h incubation at room temperature with anti-rabbit IgG conjugated to alkaline phosphatase (Sigma). Color development was performed with an alkaline phosphatase conjugate substrate kit (Bio-Rad) as described by the manufacturer.

Metabolic Labeling, Precipitation, and SDS-Polyacrylamide Gel Electrophoresis—$4–10 \times 10^6$ transfected cells were washed twice in 2 ml of methionine-deficient Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA), resuspended in 1 ml of methionine-deficient Dulbecco's modified Eagle's medium containing [^{35}S]methionine (15 μCi/10^6 cells) (ICN, Irvine, CA), and incubated at 37 °C for 6–18 h. Supernatant from the cell culture was cleared of debris by centrifugation for 5 min at $225 \times g$ at 4 °C.

To precipitate the Ig protein, 2.5 μl of rabbit anti-human Fab antiserum (R27) was added to each culture supernatant, and the supernatant was incubated at 4 °C for 2–18 h. 100 μl of IgGsorb Staph A (The Enzyme Center, Inc., Malden, MA) was then added, and the supernatant was incubated at 4 °C for 15 min to 1 h. Immune complexes were spun through a pad of 30% sucrose + 0.15% SDS + 0.5X NDET (1 × NDET = 1% Nonidet P-40, 0.4% deoxycholate, 66 mM EDTA, and 10 mM Tris, pH 7.4), and the pellet was washed sequentially in 300 μl of NDET + 0.3% SDS and 400 μl of distilled H_2O. The pellet was then resuspended in sample loading buffer (loading buffer = 25 mM Tris, pH 6.7, 2% SDS, 10% glycerol, and $\sim$0.1 μg/ml bromphenol blue) and boiled for 2 min. The samples were analyzed on polyacrylamide gels (12). For two-dimensional gel analysis, samples were first electrophoresed on 5% polyacrylamide gels (12), and the lane containing the sample of interest was excised and incubated in sample loading buffer containing 5% dithiothreitol (Boehringer Mannheim) for 20 min at room temperature. The lane was then embedded in 12.5% polyacrylamide. Electrophoresis in the second dimension was then done as described (12).

Pulse-Chase—$2–8 \times 10^6$ transfected cells/time point were washed twice in 2 ml of methionine-deficient Dulbecco's modified Eagle's medium. Washed cells were then incubated in 2 ml of methionine-deficient Dulbecco's modified Eagle's medium for 1 h at 37 °C to deplete the cells of intracellular methionine. Cells were pulsed with [^{35}S]methionine (ICN) (15 μCi/10^6 cells) for 5 min at 37 °C and then chased with 8 ml of chase medium that had been prewarmed to 37 °C (chase medium = Iscove's modified Dulbecco's medium (Irvine Scientific) + 10% bovine calf serum (Hyclone) + 3.36 mg/ml unlabeled methionine (Schwartz/Mann, Orangeburg, NY)). At various time points, 1-ml aliquots were removed to tubes containing an equal volume of prechilled phosphate-buffered saline on ice. Trichloroacetic acid-precipitable radioactivity was determined at various time points to confirm that the amount of nonradioactive methionine added was sufficient to prevent continued [^{35}S]methionine incorporation during the 3-h chase.

[1] The abbreviations used are: H, immunoglobulin heavy chain; L, immunoglobulin light chain; dansyl, 5-dimethylamino-1-naphthalenesulfonyl; PCR, polymerase chain reaction; RT, reverse transcription; UTR, untranslated region; ELISA, enzyme-linked immunosorbent assay.

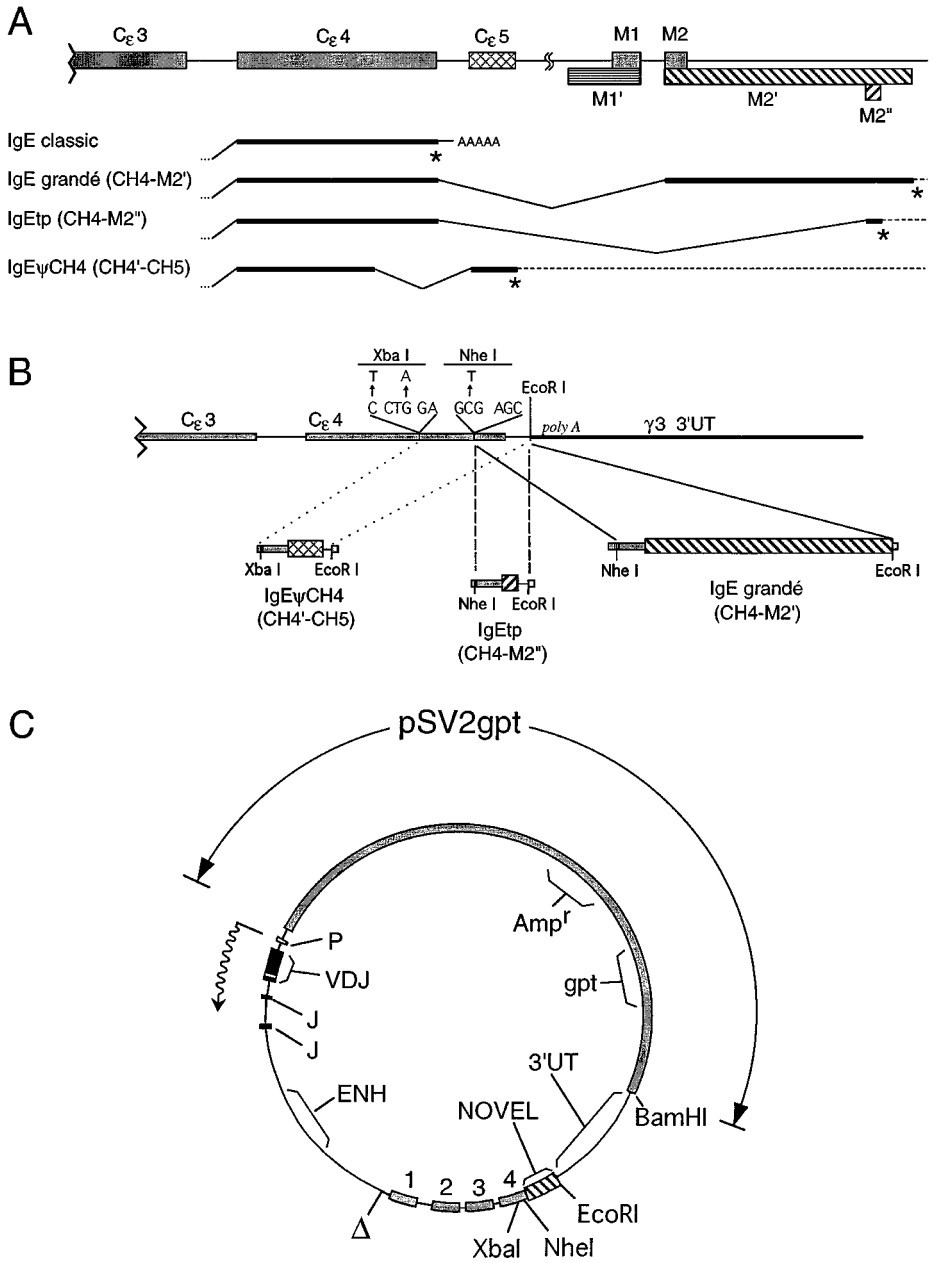

FIG. 1. *A*, structure and alternative splicing at the 3′ end of the human epsilon locus. Locations of classic membrane exons M1 and M2 and of novel exons M1′, M2′, M2″, and Cε5 are depicted at the *top* of the figure. *Below* are shown the splicing events that lead to the production of the isoforms investigated: IgE classic, IgE grandé, IgEtp, and IgEψCH4. The *dotted line* at the far 3′ end of splicing diagrams indicates uncertainty regarding downstream splicing events. *B*, strategy used to generate isoform-specific IgE constructs. The *top portion* of *B* depicts the third and fourth constant domain exons of the genomic human epsilon gene and a 3′-untranslated region, *γ3 3′-UT* (not drawn to scale), from the human γ3 immunoglobulin heavy chain locus. The mutations used to generate the *Xba*I and *Nhe*I sites in Cε4 are shown above the Cε4 exon. The *Xba*I and *Nhe*I sites in the three RT-PCR clones (at the *bottom* of the figure) were introduced with the same mutations. None of the mutations introduced amino acid substitutions. The three RT-PCR clones were then fused to the genomic exons at the *Xba*I and *Eco*RI sites or at the *Nhe*I and *Eco*RI sites. *C*, diagram of the pSV2 gpt expression vector for IgE grandé highlighting relevant features. The immunoglobulin is encoded by an anti-dansyl variable domain exon (*VDJ*), epsilon constant domain exons (*1*, *2*, *3*, and *4*), and sequence encoding the novel carboxyl terminus (*NOVEL*). Two orphan J segments are also present (*J*). Expression is driven by an Ig heavy chain promoter/leader sequence (*P*) and an Ig heavy chain enhancer element (*ENH*). The *wavy arrow* indicates the direction of transcription. *3′-UT* indicates the human γ3 3′-untranslated region. *Amp*^{r} and *gpt* indicate the β-lactamase and xanthine-guanine phosphoribosyltransferase genes used for selection in procaryotic and eukaryotic cells, respectively. The *Xba*I, *Nhe*I, and *Eco*RI sites introduced by PCR mutagenesis are shown. Also shown is the *Bam*HI site used to subclone the different constructs into expressions vectors. The *Sal*I and *Xho*I sites used to subclone into expression vectors were destroyed in the ligation and are indicated here by a Δ.

Cells were separated from the supernatant by centrifugation for 5 min at 225 × *g* at 4 °C. Cell lysates were prepared by resuspending the cell pellet in 0.5 ml of NDET, centrifuging at 4 °C for 15 min at 15,000 × *g*, and discarding the pellet. IgE was precipitated from supernatants and cell lysates with a mixture of rabbit anti-human Fab (R27 antiserum) and rabbit anti-human epsilon (ICN) as described above.

For densitometry of IgE assembly intermediates, nonreducing gels from pulse-chase experiments were visualized by autoradiography and scanned on a Hewlett/Packard ScanJet IIcx scanner. The images were analyzed at 600 dots/inch using the NIH Image software package.

RESULTS

Production of Genetically Determined IgE Isoform-specific Transfectomas—Fig. 1*B* illustrates the strategy used for generating constructs encoding specific isoforms of human IgE.

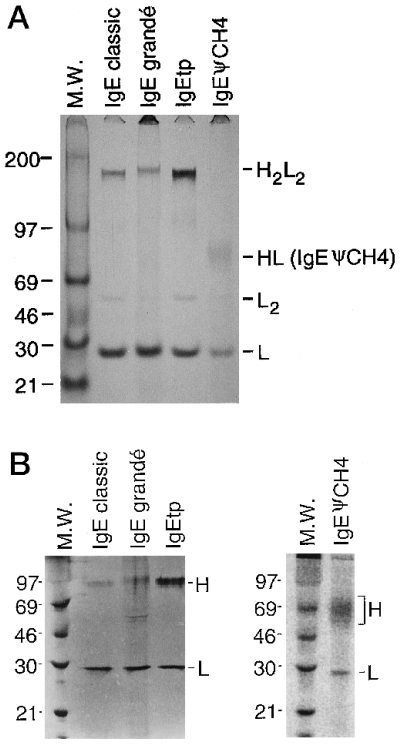

FIG. 2. **Immunoprecipitation of [^{35}S]methionine-labeled IgE isoforms.** *A*, cell lines producing IgE classic, IgE grandé, IgEtp, or IgEψCH4 were labeled 6–18 h with [^{35}S]methionine, and IgE was precipitated from the secretions using rabbit anti-human Fab followed by Staph A. Samples were loaded on a 5% gel under denaturing, nonreducing conditions. IgE classic, IgE grandé, and IgEtp are secreted as species of 190 kDa. IgEψCH4 is secreted primarily as HL, which migrates at 75–90 kDa, and free light chain of 25 kDa. *B*, samples prepared in a similar fashion were loaded onto a 12.5% gel under denaturing, reducing conditions. The 190-kDa species of IgE classic, IgE grandé, and IgEtp dissociate into two species of approximately 25 and 75 kDa upon treatment with 2-mercaptoethanol. The 75–90-kDa species of secreted IgEψCH4 migrates as light chain of approximately 25 kDa and heavy chain of 55–75 kDa after treatment with reducing agent.

RT-PCR products encompassing the 3′ portion of Cϵ4 and sequences downstream were mutagenized by PCR to introduce either an *Xba*I site or an *Nhe*I site within the Cϵ4 portion of the RT-PCR clone without altering the amino acid sequence of the encoded protein. The identical restriction sites were also engineered in a separate construct containing the Cϵ1 through Cϵ4 exons. Mutagenesis was also used to introduce an *Eco*RI site after the termination codons of the RT-PCR clones. The RT-PCR clones containing 3′ coding sequences were fused to the sequences for Cϵ1 through Cϵ4 at the *Xba*I or *Nhe*I site. A polyadenylation addition signal was provided by ligation to an *Eco*RI site previously engineered immediately upstream of the polyadenylation signal in the 3′-untranslated region from human IgG3 (*γ3 3′-UT* in Fig. 1). The resulting constructs were subcloned into pSV2 gpt containing an exon encoding a variable heavy domain specific for the hapten dansyl chloride (see Fig. 1*C*). The expression vectors were then transfected into an Sp2/0-derived cell line previously transfected with a chimeric light chain comprised of a murine anti-dansyl V_L domain and a human C_κ domain or were transfected into the J558L cell line, which synthesizes a murine λ light chain.

Analysis of the Proteins Secreted by the Isoform-determined IgE Transfectomas—Analysis of the proteins secreted by the IgE transfectomas is shown in Fig. 2. Transfectomas expressing IgE classic, IgE grandé, and IgEtp produce a protein of approximately 190 kDa (Fig. 2*A*) that reduces upon treatment with 2-mercaptoethanol to a heavy chain of approximately 75 kDa and a light chain of approximately 25 kDa (Fig. 2*B*). This indicates that these isoforms are secreted as fully assembled H_2L_2 molecules. As expected, IgE classic and IgEtp migrate with identical mobilities, whereas IgE grandé migrates slightly slower. The transfectoma expressing IgEψCH4 secretes HL, which migrates as a broad band that is 75–90 kDa as well as light chain of approximately 25 kDa under nonreducing conditions (Fig. 2*A*). A minor component migrating at approximately 150 kDa that is apparently H_2L is detectable in only some experiments (Figs. 2*A* and 3*H* and data not shown). The heavy chain of IgEψCH4 migrates somewhat faster than the heavy chain of the other three isoforms (Fig. 2*B*). This difference is not due to glycosylation because it is still evident when the transfectomas are labeled in the presence of tunicamycin, an inhibitor of *N*-linked glycosylation (data not shown). However, the diffuse migration of the 75–90-kDa species does indicate heterogeneous glycosylation of the epsilon heavy chain because it is no longer evident when the transfectoma is labeled in the presence of tunicamycin. A panel of ten transfectomas expressing IgEψCH4 (data not shown) all showed this pattern of assembly. The identities of the 75–90- and 150-kDa species as HL and H_2L, respectively, are supported by two-dimensional SDS-polyacrylamide gel electrophoresis analysis (data not shown). The species that migrate as 75–90 and 150 kDa in the first dimension (nonreducing conditions) dissociate into heavy and light chains when run reduced in the second dimension. Furthermore, the 75–90-kDa species both immunoprecipitates and reacts in Western blots with epsilon-specific monoclonal and polyclonal reagents (data not shown). In addition to fully assembled H_2L_2 molecules, all four cell lines secrete free light chain (L) and light chain dimers (L_2), as is frequently observed in this expression system (Fig. 2*A*). Secretion of light chain dimer is not evident for the IgEψCH4-producing transfectoma in Fig. 2*A* but can be seen occasionally (*e.g.* Fig. 3*H*).

Because the protein secreted by the cell line producing IgE grandé does not migrate as slowly as one would predict based on amino acid translation, we undertook experiments to verify that the protein was indeed complete and intact. Initially, the expression construct for IgE grandé was subjected to extensive restriction analysis, which showed that the entire coding region had been retained in the construct (data not shown). Due to concern that the novel 136-amino acid tail of the IgE grandé could be post-translationally cleaved, the protein was analyzed by ELISA and Western blotting using antibodies specific for the Cϵ2/Cϵ3 boundary (CIA-7.12) and for the COOH-terminal ten amino acids of IgE grandé (α-2331). As expected, both IgE classic and IgE grandé were recognized by CIA-7.12, whereas α-2331 recognized IgE grandé but failed to recognize classic secreted IgE (Table I and data not shown). Neither CIA-7.12 nor α-2331 recognized an IgG control. Recognition of IgE grandé by α-2331 indicates that the large secreted terminus of IgE grandé is not removed by proteolytic processing. In addition, recognition of IgE grandé by monoclonal antibody CIA-7.12 indicates that the Cϵ2-Cϵ3 interface (the epitope that CIA-7.12 recognizes) is intact; the ability of IgE grandé to bind antigen confirms that V_H is present.

IgE Isoform Assembly and Secretion—To dissect the assembly pathways of the various forms of IgE and to determine the efficiency of secretion, we conducted pulse-chase experiments. As shown in Fig. 3*A*, the classic secreted form of IgE is assembled first to an HL intermediate that is abundant as early as 5 min post-chase. It is later assembled into the H_2L_2 form that is detectable by 10 min post-chase and abundant within the cells by 20 min post-chase. Very similar kinetics are observed for the assembly of IgE grandé (Fig. 3*C*) and IgEtp (Fig. 3*E*). IgE

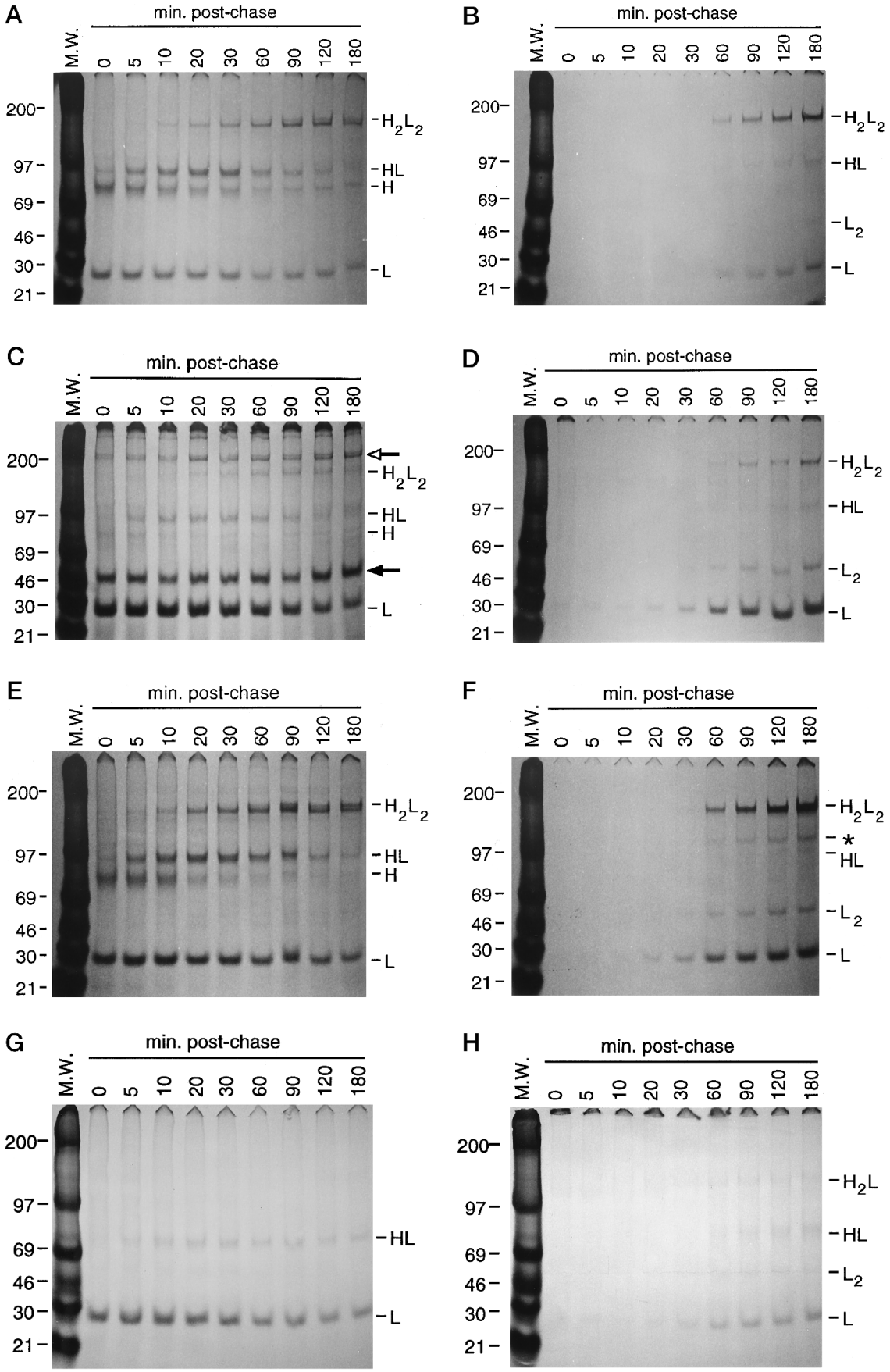

FIG. 3. **Intracellular assembly and secretion of IgE isoforms.** 2–8 × 10^6 cells/time point were incubated in methionine-deficient medium for 1 h to deplete intracellular methionine. Cells were pulsed with [^{35}S]methionine (15 μCi/10^6 cells) for 5 min at 37 °C and then chased with a ~100-fold excess of unlabeled methionine. Samples were taken at various time points following addition of the chase. Epsilon and kappa chains were then immunoprecipitated from the cytoplasms and secretions at each time point and run on 5% gels under nonreducing conditions. *A*, IgE

TABLE I
Recognition of IgE classic and IgE grandé by anti-constant region and anti-IgE grandé antibodies

	Plates coated with[a]		Detecting reagent[a]		$A_{410\ nm}$[a,d]		
	DNS-BSA	CIA-7.12[b]	CIA-7.12[b]	α-2331[c]	IgE classic	IgE grandé	IgG
EXPT 1	+	−	+	−	2.676	0.452	0.004
EXPT 2	−	+	+	−	2.697	0.588	0.002
EXPT 3	−	+	−	+	0.061	1.046	0.065

[a] IgE classic, IgE grandé, or IgG were captured on ELISA plates coated with either dansyl chloride-bovine serum albumin (DNS-BSA) or an anti-IgE monoclonal antibody (CIA-7.12) and then detected with either anti-IgE monoclonal antibody (CIA-7.12) or with an antiserum specific for a sequence unique to IgE grandé (α-2331). The data shown are the average results of duplicate wells.
[b] Murine monoclonal antibody recognizing the epsilon heavy chain constant 2/constant 3 domain boundary.
[c] Rabbit antiserum generated against a peptide corresponding to the COOH-terminal 10 amino acids of IgE grandé.
[d] Secondary antibodies were conjugated to alkaline phosphatase. The substrate was *p*-nitrophenyl phosphate.

grandé and IgEtp also assemble through an HL intermediate. In addition, for IgEtp, small quantities of H_2 and H_2L are observed intracellularly (Fig. 3*E*), and some H_2 may be present in the secretions (*band* marked with *asterisk* in Fig. 3*F*). These assembly intermediates are minor components of the secreted protein and are below the level of detection in Fig. 2. In all experiments conducted with IgE classic, IgE grandé, and IgEtp, the intracellular H_2L_2 form migrates as a doublet (Fig. 3, *A*, *C*, and *E*), probably representing IgE at various stages of glycosylation, as the H_2L_2 form in the secretions migrates as a single band (Fig. 3, *B*, *D*, and *F*). Pulse-chase experiments conducted with IgEψCH4 show the only detectable species in the cytoplasm to be light chain and species that migrate as HL half-molecules of IgEψCH4 (Fig. 3*G*). No free heavy chain is visible, and there is very little assembly to the H_2L_2 form.

Fully assembled IgE classic, IgE grandé, and IgEtp are detectable in the secretions starting at 60 min post-chase (Fig. 3, *B*, *D*, and *F*). Quantitative analysis of the gels shown in Fig. 3 indicates that in the case of IgE classic and IgE grandé, approximately 25% of the total Ig produced during the 5-min pulse is secreted as H_2L_2 during the course of the experiment (Fig. 4*A*). Following reduction, the secreted proteins migrate as heavy and light chains of approximately 75 and 25 kDa (Figs. 2*B* and 5 and data not shown). IgEtp is the most efficiently secreted of the isoforms examined, with approximately 40% of the Ig secreted as H_2L_2 during the course of the experiment (Fig. 4*A*). HL is secreted in varying amounts for each of the isoforms examined (Fig. 3, *B*, *D*, *F*, and *H*). Secretion of small amounts of HL was also reported for IgE classic in the recent study by Batista *et al.* (8). In the case of IgEψCH4 (Fig. 3*H*), the HL form constitutes the majority of IgE secreted by the transfectoma, with a smaller amount secreted as H_2L. The efficiency of secretion of these two forms is shown in Fig. 4*B* and indicates that by 3 h after the pulse, approximately 20% of the labeled Ig is secreted in HL form, whereas only ~10% is secreted as H_2L. All four transfectants synthesize excess light chain that is secreted as free L and as L_2 dimers (Fig. 3, *B*, *D*, *F*, and *H*).

Two species of 50 and 200 kDa (*closed* and *open arrows* in Fig. 3*C*) co-precipitate with intracellular IgE grandé and (to a lesser extent) IgEtp (Fig. 3*E*). The mobility of these two coprecipitating proteins is not affected by treatment with 2-merceptoethanol (Fig. 5, *cytoplasm*). It is also noteworthy that the 200-kDa species is present at zero time. From these data, we conclude that the 200- and 50-kDa proteins are not assembly intermediates of IgE but instead represent non-IgE proteins that are coprecipitated. The 200-kDa protein is not secreted, although a band at ~50 kDa is seen in the secretions (Fig. 3, *B*, *D*, and *F*). However, the latter is no longer detectable after treatment with 2-merceptoethanol (Fig. 5, secretion) and most

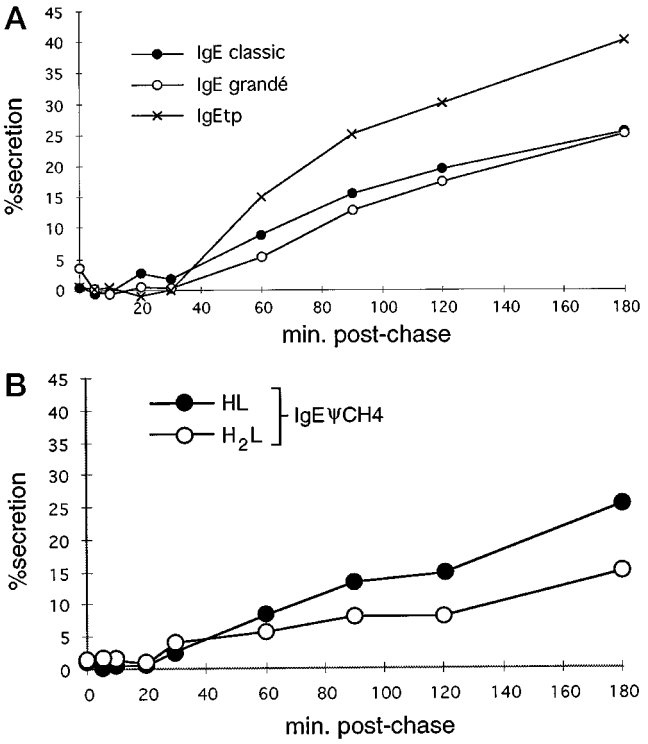

FIG. 4. **Kinetics of secretion of IgE isoforms.** Data are from densitometric analysis of the pulse-chase experiments shown in Fig. 3. *A*, for IgE classic, IgE grandé, and IgEtp, the amount of H_2L_2 secreted is expressed as a percentage of the total heavy and light chain produced during the pulse. *B*, for IgEψCH4, the amount of H_2L and HL secreted is expressed as a percentage of the total heavy and light chain produced during the pulse.

probably represents light chain dimers (L_2). Thus, the co-precipitating 50-kDa cytoplasmic protein, like the 200-kDa protein, does not appear to be secreted.

These findings are in marked contrast to those of Batista *et al.* (8), who concluded that the novel isoforms IgE grandé (CH4-M2′) and IgEψCH4 (also designated CH4′-CH5 and CH4′-I) are not secreted by plasma cells. One difference between the present and earlier studies is the murine myeloma cell line used for expression. To determine if the cell lines could account for the different results, we transfected our expression vector for IgE grandé into J558L, the murine myeloma cell line used by Batista *et al.* No positive clones were identified when cell culture supernatants of several hundred selection-resistant transfectomas were screened by ELISA (data not shown). However, several clones demonstrating high levels of intracellular epsi-

classic cytoplasms. *B*, IgE classic secretions. *C*, IgE grandé cytoplasms. *D*, IgE grandé secretions. *E*, IgEtp cytoplasms. *F*, IgEtp secretions. *G*, IgEψCH4 cytoplasms. *H*, IgEψCH4 secretions. The *open* and *closed arrows* in *C* denote non-IgE proteins co-precipitating with IgE grandé; the *asterisk* in *F* denotes a secreted species that we propose but have not proven to be the H_2 form of IgEtp.

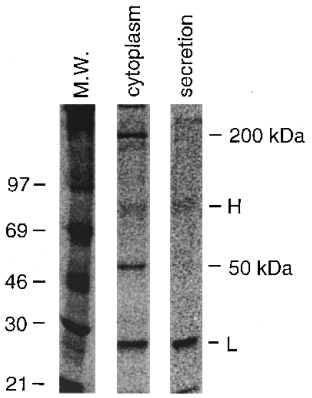

FIG. 5. **Migration of 50- and 200-kDa co-precipitating species from the cytoplasm is unaffected by treatment with reducing agent.** Cytoplasmic and secretion samples from the IgE grandé pulse-chase experiment were reduced with 2-mercaptoethanol. The IgE grandé in both cytoplasm and secretion reduces to a heavy chain of ~75 kDa and a light chain of ~25 kDa. The co-precipitating species from the cytoplasm, however, still migrate as 50 and 200 kDa following reduction. The 50-kDa light chain dimer in the secretions (see Fig. 3, *B*, *D*, and *F*) reduces to light chain.

lon chain were identified by ELISA using anti-IgE to capture epsilon chain from cell lysates and alkaline phosphatase-conjugated anti-IgE to detect bound epsilon chain. Pulse-chase analysis indicates that large amounts of epsilon chain are produced by this cell line but are degraded intracellularly. Fig. 6*A* shows that the IgE grandé is assembled in J558L into an HL form that is seen by 5 min post-chase. However, assembly appears to stop at this intermediate form, and very little H_2L_2 is formed. A band that migrates slightly faster than the H and HL forms is seen and may represent a degradation product. When these samples were electrophoresed under reducing conditions and analyzed by densitometry, the total intracellular epsilon chain was found to decrease to less than 5% of the original level by 180 min post-chase (data not shown). The reduced samples also demonstrate the existence of additional, labile species that likely represent degradation products. No H_2L_2 is detectable in the supernatant of the J558L transfectoma during the same interval, although small quantities of HL may appear in the supernatant (Fig. 6*B* and data not shown). When selection-resistant transfectomas of J558L were screened by ELISA of cell lysates, a large percentage (~50%) of the clones were positive for intracellular epsilon chain, but none secreted a detectable amount of IgE. Therefore, the intracellular degradation of IgE grandé appears to occur whenever it is expressed in J558L. These results demonstrate that different cell lines can vary in their ability to assemble and secrete the IgE isoforms.

DISCUSSION

Secreted IgE functions via its ability to bind to specific IgE receptors. These receptors make it possible for IgE to act as a very sensitive trigger for initiating both afferent and efferent immune reactivity in the presence of low doses of antigen. Three such "receptors" have been identified; the high affinity IgE receptor (FcεRI), the low affinity IgE receptor (FcεRII or CD23), and galectin 3 (formerly known as epsilon-binding protein) (13).

IgE mediates immediate type hypersensitivity primarily through its association with the high affinity IgE receptor present on the surface of mast cells and basophils. These cells release a variety of soluble mediators upon cross-linking of their receptor-bound IgE by a cognate antigen. There is also evidence that IgE bound to the high affinity receptor on mast cells can be cross-linked by member(s) of a broad class of

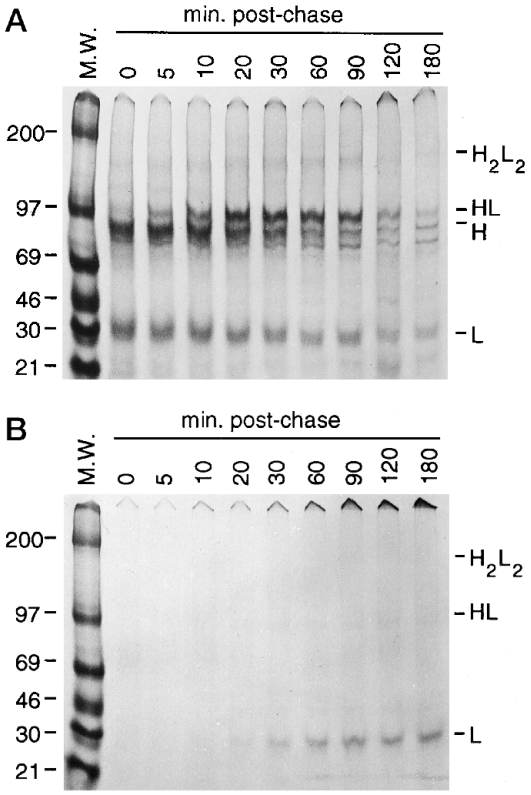

FIG. 6. **The J558L myeloma cell line does not assemble or secrete IgE grandé.** Pulse-chase analysis was done as described in the legend to Fig. 3. *A*, cytoplasms. *B*, secretions.

IgE-dependent histamine releasing factors (14). Studies involving histamine releasing factors have led to the suggestion of a functional heterogeneity of IgE. The basis for this heterogeneity is not understood but has been speculated to be the result of differential glycosylation (14). IgE has also been suggested to participate in a variety of other immune processes such as antigen recognition, antibody-dependent cellular cytotoxicity, and B cell growth via binding to the high affinity IgE receptor (FcεR I), the low affinity IgE receptor (FcεR II), or galectin 3. The existence of splice variants of IgE provide an additional possible explanation for the functional heterogeneity of IgE and suggests that IgE may differ in its primary protein structure as well as glycosylation. Splice variants have also been observed in the mRNA of human IgA (15) and avian IgY (16).

The low serum levels of IgE and the similar molecular size predicted for many putative splice variants make purification of the individual protein isoforms of IgE from serum problematic. Our approach using recombinant DNA transfection has the advantage that vectors can be constructed that encode a single isoform of IgE, guaranteeing the homogeneity of the isoforms produced. In the current study, we have constructed vectors to express four isoforms of IgE in an Sp2/0-derived murine cell line. The kinetics and efficiency of the assembly and secretion are similar for each of these isoforms. The results indicate that of the four secreted isoforms examined, IgE classic, IgE grandé, and IgEtp are fully assembled by the Sp2/0 cells, whereas IgEψCH4 is secreted predominately as HL half-molecules. The incomplete assembly of IgEψCH4 is not entirely unexpected given that the constant regions of Igs are stabilized by noncovalent interactions between the COOH-terminal domains of both heavy chains. In the IgEψCH4 isoform, the 3′ portion of the Cε4 exon is removed by splicing from a cryptic splice donor within that exon and replaced by sequence from the Cε5 cryptic exon. It is noteworthy that among the residues

removed from IgEψCH4 is a cysteine that is universally conserved in immunoglobulin domains (5). The domain structure of IgEψCH4 is therefore likely to be disrupted. Speculation as to the physiological relevance of this structural variation must await a detailed functional comparison of these IgE isoforms.

In previous studies, we have reported that IgE grandé is detectable in the supernatant of the IgE-producing cell line AF-10, an IgE-stable, mycoplasma-free subclone of U266 (4) in the serum of a patient with an IgE myeloma and in the serum of highly atopic persons with very high serum levels of IgE (3, 4). Because IgE grandé is the only known isoform with an M_r sufficiently different from IgE classic to resolve by SDS-polyacrylamide gel electrophoresis, it is impossible to ascertain from the previous data whether the other described isoforms were also present in the U266/AF-10 supernatant or the serum IgE from myeloma or highly atopic patients, although heterogeneity of bands in the appropriate size range was evident (3). In a recent study by Batista *et al.* (8), Western analysis and immunoprecipitation of the supernatant of U266 showed two species similar in size to the epsilon chain. However, treatment with glycosidase PNGase F caused the two bands to be reduced in size and comigrate as a single species, and it was concluded that only one isoform of IgE (*i.e.* the "classic secreted" isoform) is secreted by B cells (8). The same study had found that when the murine myeloma cell line J558L was transfected with expression vectors encoding individual splice variants of epsilon heavy chain, the recombinant epsilon chains could be detected in the cytosol of the transfectants but not in the secretions.

Secretion of the four isoforms of IgE reported in the present study is clear. In light of the aforementioned report, this raises questions concerning cell line-dependent factors affecting protein expression. Batista *et al.* (8) expressed their isoform-determined IgE genes in the J558L myeloma cell line using the pRc/CMV expression vector, whereas we expressed the proteins in the Sp2/0 myeloma cell line using the pSV2 gpt expression vector. Because adequate levels of epsilon chain were evident in the cytoplasm of the transfectants in both studies, it is unlikely that the different expression vectors account for the difference in secretion. Indeed, when we expressed IgE grandé in J558L using the same pSV2 gpt-based expression vector, the amount of epsilon heavy chain produced by the J558L transfectant was much greater than that produced by Sp2/0, but the J558L transfectant fails to efficiently assemble and secrete it (Fig. 3, *C* and *D*, and Fig. 6, *A* and *B*). Quantitation of the epsilon heavy chains on a 12.5% gel under reducing conditions (not shown) indicates that nearly all of the epsilon chain produced by these cells during the 5-min pulse is degraded within 180 min. Although the Sp2/0 IgE grandé transfectoma produces far less epsilon chain than its J558 counterpart, readily detectable levels of IgE (~25% of the epsilon and kappa chains labeled during the 5-min pulse) are secreted in the H_2L_2 form by 180 min post-chase, and there is no evidence for heavy chain degradation (Fig. 4 and data not shown). Additionally, the kinetics of assembly and secretion of the IgE grandé is very similar to that of IgE classic and IgEtp (Figs. 3 and 4*A*).

Several factors could cause cell line-dependent variation in protein secretion. One such factor is glycosylation. We have some evidence[2] that IgE grandé is degraded when the producing cell line is labeled in the presence of tunicamycin. Glycosylation-dependent differences in post-translational proteolytic processing have been described for the soluble form of CD23 (17). Also, cell line-dependent variation has been described in the utilization of *N*-linked carbohydrate addition sites.[3] It is possible that the altered exon usage of some IgE isoforms alters the accessibility of certain addition sites to some glycosylases-glycosidases and that some cell lines are better able to process these carbohydrate addition sites in their altered molecular context. Another possible explanation lies in chaperone proteins. It has been shown that during Ig assembly, Igs interact in a sequential fashion with at least two chaperones, BiP and GRP94 (18). Although we do not observe co-precipitation of either of these two chaperones with IgE under the conditions used, it is interesting to note that both IgE grandé and IgEtp are seen to co-precipitate with two species of ~50 and ~200 kDa (Figs. 3 and 5). We have as yet taken no steps to identify these proteins; however, they have been observed to co-precipitate with other antibody isotypes including IgA.[4]

We have shown that the protein products of three novel messages for human IgE are translated by the murine myeloma Sp2/0 and are efficiently assembled and secreted. Alternatively spliced mRNAs have been described for one more potentially secreted epsilon protein (5–7) as well as two forms of membrane epsilon chains. It is quite likely that all the described forms of epsilon mRNA are expressed at the protein level.

On the basis of the present and previous studies, it appears that human IgE is comprised of a family of proteins generated by alternative RNA splicing. Individual secreted members of this family may have some unique properties as circulating, cytophilic Igs and may differ in their ability to carry out IgE-mediated functions through binding to FcϵRI, FcϵRII, or galectin 3. Although the contact residues for the former two receptors appear to be intact in the secreted epsilon isoforms, the isoforms may nevertheless function differently because the CH4 domain may be critical in constraining the three-dimensional shape of the IgE constant region. IgEψCH4 merits special attention because this isoform is secreted primarily as HL half-molecules (Figs. 2*A* and 3*H*), although we have not ruled out noncovalent interactions between half-molecules. If IgEψCH4 binds to FcϵR I as a half-molecule, it would be less able to cross-link the receptors. It may therefore be less able to arm mast cells and basophils for antigen-triggered release and may in fact inhibit the release. Our findings gain added significance in that the relative levels of mRNAs encoding various isoforms are altered in allergic and parasitic diseases (3). Additionally, we have demonstrated a cell line-dependent variability in the efficiency of assembly and secretion of IgE grandé, suggesting that additional, as yet uncharacterized regulatory mechanisms of intracellular IgE assembly and/or trafficking may exist. The availability of highly purified IgE protein isoforms will now allow us to determine if the different isoforms have unique functional properties.

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