

Competing Functions Encoded in the Allergy-Associated *FcεRI/β* Gene

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Summary

Allergic reactions are triggered via crosslinking of the high-affinity receptor for immunoglobulin E, *FcεRI*. In humans, *FcεRI* is expressed as a tetramer ($\alpha\beta\gamma_2$) and a trimer ($\alpha\gamma_2$). The β subunit is an amplifier of *FcεRI* surface expression and signaling. Here, we show that as a consequence of alternative splicing, the *FcεRI/β* gene encodes two proteins with opposing and competing functions. One isoform is the full-length classical β , the other a novel truncated form, β_T . In contrast to β , β_T prevents *FcεRI* surface expression by inhibiting α chain maturation. Moreover, β_T competes with β to control *FcεRI* surface expression in vitro. We propose that the relative abundance of the products of the β gene may control the level of *FcεRI* surface expression and thereby influence susceptibility to allergic diseases.

Introduction

FcεRI, the high-affinity cell surface receptor for the Fc portion of IgE, is a multimeric complex expressed on basophils, mast cells, and antigen-presenting cells in humans (Kinet, 1999). On mast cells and basophils, *FcεRI* aggregation by multivalent antigen causes cell activation, which proceeds along multiple signaling pathways that ultimately cause allergic mediator secretion and cytokine gene transcription (Turner and Kinet, 1999). On antigen-presenting cells, *FcεRI* aggregation leads to antigen internalization and processing, which results in efficient presentation by MHC molecules (Maurer et al., 1996).

The *FcεRI* complex is expressed exclusively as a tetramer on rodent cells but exists as a tetramer or trimer on human cells. The tetrameric form is expressed on mast cells and basophils and is composed of an α subunit, a β subunit, and a disulfide-linked γ subunit dimer ($\alpha\beta\gamma_2$). Complexes lacking the β subunit ($\alpha\gamma_2$) are found

on human Langerhans cells, dendritic cells, and monocytes (Bieber et al., 1992; Maurer et al., 1996, 1994; Wang et al., 1992). A correlation thus exists between the tetrameric isoform and allergy mediator release, as well as between the trimeric isoform and antigen presentation. Whether this correlation is due to a difference in expression and signaling capacity between the two isoforms has not been investigated.

The α subunit of the *FcεRI* complex is a type I membrane protein comprised of two extracellular immunoglobulin (Ig)-like domains and a short intracellular tail. The α chain is heavily N-glycosylated. Approximately 50% of the mature α chain molecular weight is derived from glycosylation. This chain is responsible for binding IgE (Wurzburg and Jardetzky, 2002). The β chain spans the membrane four times such that the amino and carboxy termini are intracellular (Holowka and Metzger, 1982). An atypical ITAM is found near the carboxy terminus of β . This ITAM is anomalous in that it contains an extra tyrosine and a shortened spacer region. The β ITAM does not appear to engage in appreciable signal transduction in and of itself (Jouvin et al., 1994; Shiue et al., 1995; Wilson et al., 1995). The main signaling subunit of *FcεRI* is the γ chain dimer. The γ subunit is a type I membrane protein with a short extracellular portion and a long intracellular tail containing an ITAM. It is homologous to the ζ chain of the T cell receptor.

Comparison of the two forms of *FcεRI* on human cells has revealed a dual amplifier role for the β chain. Cells expressing the tetrameric form, $\alpha\beta\gamma_2$, display increased signaling capacity and increased cell surface expression of *FcεRI* as compared to cells expressing the trimeric form, $\alpha\gamma_2$ (Dombrowicz et al., 1998; Donnadieu et al., 2000b; Lin et al., 1996; Saini et al., 2001). The mechanism of the signaling amplification in the presence of β is due to increased phosphorylation of γ and recruitment of syk after receptor aggregation. This enhancement appears to be due to the association of signaling proteins such as Lyn with β under resting conditions and after receptor aggregation (Jouvin et al., 1994; Kihara and Siraganian, 1994; Kimura et al., 1996; Vonakis et al., 1997).

With regard to the amplification of cell surface expression, it has been demonstrated that β increases the intracellular processing of the α chain (Donnadieu et al., 2000b). Newly synthesized α chain is core-glycosylated in the endoplasmic reticulum (ER). This glycosylation is required for proper folding of the α chain in the ER and for subsequent cell-surface expression (Letourneur et al., 1995). This immature α is then transferred to the Golgi where terminal glycosylation occurs, such that the core mannose-rich sugars are replaced with complex, heterogeneous sugars. In $\alpha\beta\gamma_2$ -expressing cells, the relative amount of mature α is substantially higher than in $\alpha\gamma_2$ expressors for the same level of α chain synthesis. It would, therefore, appear that β chain acts as a chaperone for transporting the *FcεRI* complex to the cell surface.

Given the capacity of β to act as an amplifier for *FcεRI* signaling and expression, it follows that the β chain may

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play a role in atopy. This hypothesis is supported by various genetic analyses (reviewed in Cookson, 2002). Population studies have found a linkage between human chromosomal region 11q13 and asthma and allergic diseases (Collee et al., 1993; Cookson, 1999; Cookson et al., 1989; Dizier et al., 2000; Folster-Holst et al., 1998; Hizawa et al., 1998; Moffatt and Cookson, 1999; Palmer et al., 1998; Shirakawa et al., 1994a; van Herwerden et al., 1995). The *FcεRIβ* gene is also found within 11q13. Moreover, polymorphisms of the *FcεRIβ* gene have been reported to be associated with asthma and allergic diseases (Adra et al., 1999; Cookson, 1999; Green et al., 1998; Hijazi et al., 1998; Hill and Cookson, 1996; Hill et al., 1995; Laprise et al., 2000; Nagata et al., 2001; Rigoli et al., 2000; Shirakawa et al., 1994b). The mechanism by which these polymorphisms may contribute to the pathogenesis of these diseases remains unknown (Donnadieu et al., 2000b). In our search for additional polymorphisms in the β gene, we have identified an isoform of β . We show here that this isoform represents an alternate splice product of the β gene. The corresponding protein is produced and reveals a function that is the opposite of classical β . Whereas classical β is an amplifier of *FcεRI* function and expression, the variant protein is a negative regulator of *FcεRI* expression.

Results

Identification of an Alternative Splice Product of the β Gene, β_T

Given the strong candidacy of *FcεRIβ* as a risk factor for allergic diseases, it is important to identify potentially relevant polymorphisms. A number of associated point mutations have already been described, yet the mechanism by which they increase susceptibility to atopy remains unknown (Donnadieu et al., 2000a). In the process of screening for additional polymorphisms of the *FcεRIβ* gene in humans, we have identified a novel β chain transcript. Basophils were semipurified from blood obtained from individuals in the general population. RNA was extracted, reverse-transcribed (RT), and amplified by polymerase chain reaction (PCR) using a forward primer located within exon 2 and a reverse primer located within exon 7 of the *FcεRIβ* gene (Kuster et al., 1992). Amplification produced the anticipated 700 bp band corresponding to the fully spliced message as well as an unexpected band of 1.1 kb. Both transcripts were also present in human cord blood-derived mast cells (CBMC), but not in the human monocytic cell line U937, which does not express β (Figure 1A). Sequencing of the 1.1 kb band identified it to result from retention of the 5th intron in mature β message (Figure 1B) (Kuster et al., 1992). The novel transcript, therefore, represents an alternative splice product of the β gene. The predicted translation product of the newly identified transcript would be truncated due to the presence of an in frame stop codon within the sequence contributed by the intron (Figure 1B). The transcript and resultant protein will thus be referred to as β_T .

In order to verify that the detection of β_T was not a PCR artifact, RNase protection assays (RPA) were performed. Transfectants were generated with α and γ cDNAs plus either β or β_T cDNA. These transfectants

were used as controls for the β or the β_T protected fragments. The antisense β probe used in this analysis extended from the end of exon 4 through exon 5 and up to the beginning of intron 5. Hybridization with classical β message should protect 171 nucleotides (nt) of the labeled probe, whereas hybridization with β_T message should protect 212 nt. Protected fragments of the expected length for β and β_T were detected in $\alpha\beta\gamma_2$ and $\alpha\beta_T\gamma_2$ transfectants, respectively, but not in untransfected U937 cells, thus confirming the specificity of the probe as well as the sizes of the protected fragments (Figure 1C). RPA analysis on RNA derived from CBMC revealed that both β transcripts were endogenously produced and coexpressed. Mouse equivalents of β and β_T were detected by RPA in the mouse basophilic cell line C1.MC/C57.1 (C57) with the corresponding mouse probe, indicating that β_T synthesis is not limited to humans (Figure 1C).

As aforementioned, the protein corresponding to the β_T message would be truncated due to the presence of an in-frame stop codon within intron 5. Specifically, the fourth transmembrane region and C-terminal intracellular tail of classical β would be deleted and replaced with 16 novel amino acids encoded at the beginning of intron 5 (Figure 1B). To demonstrate that the β_T message is indeed translated into a protein, lysates from CBMC and from the human basophilic cell line KU812 were immunoprecipitated and immunoblotted with an anti-human β antibody (Donnadieu et al., 2000b; Maurer et al., 1996). This antibody is directed against the β N terminus, which is common between the two transcripts. Lysates from the $\alpha\beta\gamma_2$ and $\alpha\beta_T\gamma_2$ transfectants were processed in an identical fashion and used as controls. As expected, a band of 28 kDa corresponding to β was immunoprecipitated and immunoblotted from the lysate of the $\alpha\beta\gamma_2$ transfectants and KU812, but not from the $\alpha\beta_T\gamma_2$ transfectants. A protein of the predicted size (21.4 kDa) for β_T was present in KU812 and $\alpha\beta_T\gamma_2$, but not in $\alpha\beta\gamma_2$ transfectants. Both the truncated protein and full-length protein were also present in CBMC (Figure 1D). These data are further evidence that β and β_T are endogenously produced and coexpressed in a human basophilic cell line as well as in primary human mast cells.

β_T Functions as a Negative Regulator of *FcεRI* Surface Expression

Having established that β_T protein is endogenously produced, the next step was to determine its function. Since β is an amplifier of *FcεRI* expression and signaling, the effect of β_T on cell surface expression was tested (Dombrowicz et al., 1998; Donnadieu et al., 2000b; Lin et al., 1996). The transfection strategy previously used to demonstrate the expression amplification function of β was applied here (Donnadieu et al., 2000b; Lin et al., 1996). A number of $\alpha\gamma_2$, $\alpha\beta\gamma_2$, and $\alpha\beta_T\gamma_2$ stable transfectants were established in U937 cells and in the mouse fibroblastic cell line NIH 3T3. The entire panel of clones was then compared for *FcεRI* cell surface expression. The cells were stained with IgE, and surface expression was quantified by flow cytometry. In accordance with the previous study, $\alpha\beta\gamma_2$ clones expressed a higher level of *FcεRI* than did $\alpha\gamma_2$ clones (Donnadieu et al., 2000b). Surprisingly, the clones containing β_T expressed fewer

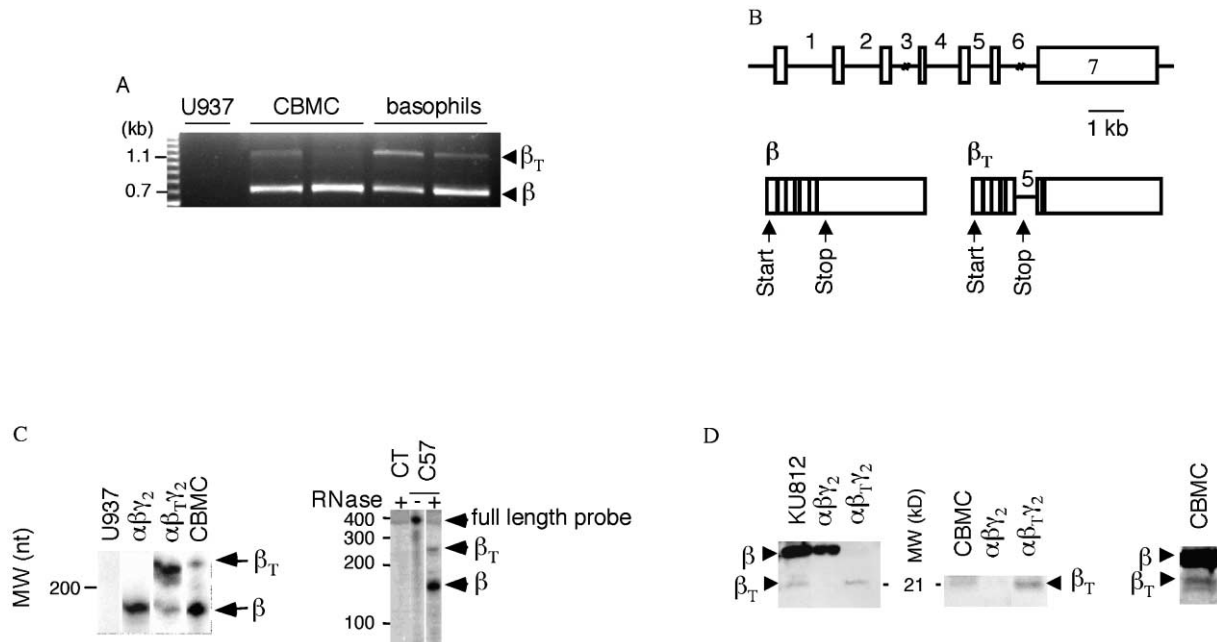


Figure 1. Detection of the β_T Message and Protein in Mast Cells and Basophils

(A) Detection of β_T message by RT-PCR in human basophils (basophils; 2 donors) and cord blood mast cells (CBMC; 2 donors), but not in a non- β expressing cell line (U937).

(B) Schematic representation of the β gene (upper panel) and the β and β_T messages (lower panels). Numbers represent individual introns. β and β_T use the same ATG located at nt 45 (exon 1). The stop codon in β is located at nt 776 (exon 7). The β_T message is identical to β up to the end of exon 5 (nt 581). In β_T , intron 5 is retained between exon 5 and exon 6. As a result, β_T is 402 nt longer. An in-frame stop codon is present after 48 nt in intron 5. Retention of intron 5 results in a shorter protein in which the fourth transmembrane domain and the C-terminal tail are replaced with a novel 16 amino acid peptide.

(C) Detection of β_T message by RNase protection assay in CBMC and the mouse basophil cell line C57. (Left panel) The expected fragment of 212 nt for human β_T is detected in CBMC and $\alpha\beta\gamma_2$ transfectants, but not in untransfected U937 or in $\alpha\beta\gamma_2$ transfectants. The expected fragment of 171 nt for human β is detected in CBMC and $\alpha\beta\gamma_2$ transfectants, but not in untransfected U937 or in $\alpha\beta\gamma_2$ transfectants. The lower band in the $\alpha\beta\gamma_2$ lane is a nonspecific degradation product. (Right panel) RNA from the mouse mast cell line CLMC/C57.1 (C57), but not mouse liver RNA (CT), contains both protected mouse β_T (266 nt) and β (163 nt) fragments. The full-length mouse probe is detected when RNase treatment is omitted (RNase -).

(D) Detection of the β_T protein in CBMC (20 E6 cells/lane) and in the human basophil cell line KU812 (50 E6 cells/lane). Cells were treated with the proteasome inhibitor ALLN and lysed. β and β_T were immunoprecipitated and Western blotted with an anti- β antibody directed against the common N-terminal tail. $\alpha\beta\gamma_2$ and $\alpha\beta_T\gamma_2$ transfectants (10 E6 cells/lane upper panel, 20 E6 cells/lane lower panel) are used as controls for β and β_T , respectively.

surface receptors than the $\alpha\beta\gamma_2$ clones ($p = 4.5 \times 10^{-6}$) and notably less than the $\alpha\gamma_2$ clones as well ($p = 1.5 \times 10^{-3}$) (Figure 2). Similar results were obtained with NIH 3T3 stable transfectants (data not shown). The lower expression levels in $\alpha\beta_T\gamma_2$ as compared to $\alpha\gamma_2$ clones highlight the fact that β_T does not simply lack the expression amplification function associated with β , but actively prevents receptor expression. Furthermore, KU812 cells were stably transfected with α and γ cDNA encoding constructs. Transient transfection with β and β_T cDNA caused a 20% reduction in the FcεRI MFI as compared to transfection with β cDNA alone (data not shown). This indicates that β_T can act as a negative regulator in a human basophil cell line as well and can compete with the function of β .

β_T Prevents α Maturation

The mechanism of the apparent inhibitory action of β_T on FcεRI surface expression was investigated. Classical β amplifies receptor expression by facilitating the intracellular processing and maturation of the α chain (Don-

nadiou et al., 2000b). Considering that β_T appears to inhibit receptor expression, it stood to reason that perhaps β_T interferes with α maturation. Newly synthesized α chain is core-glycosylated in the ER. It migrates as a band of about 46 kDa on SDS PAGE. This immature α is endoglycosidase H (Endo H) sensitive and results in a band of about 30 kDa on SDS PAGE following Endo H treatment. This is the expected molecular weight for the core protein. Immature α is then transferred to the Golgi where terminal glycosylation occurs, such that the core mannose-rich sugars are replaced with complex, heterogeneous sugars that are Endo H resistant. Mature α appears as a smear around 66 kDa (Letourneur et al., 1995). When $\alpha\gamma_2$ and $\alpha\beta\gamma_2$ transfectants with the same level of surface FcεRI expression are compared, the amount of immature α chain is much greater in the $\alpha\gamma_2$ than in the $\alpha\beta\gamma_2$ clones. This indicates that α maturation is considerably improved in the presence of β chain. Verily, the effect on α maturation may be the primary mechanism by which β amplifies FcεRI surface expression (Donnadiou et al., 2000b). The effect of β_T on α

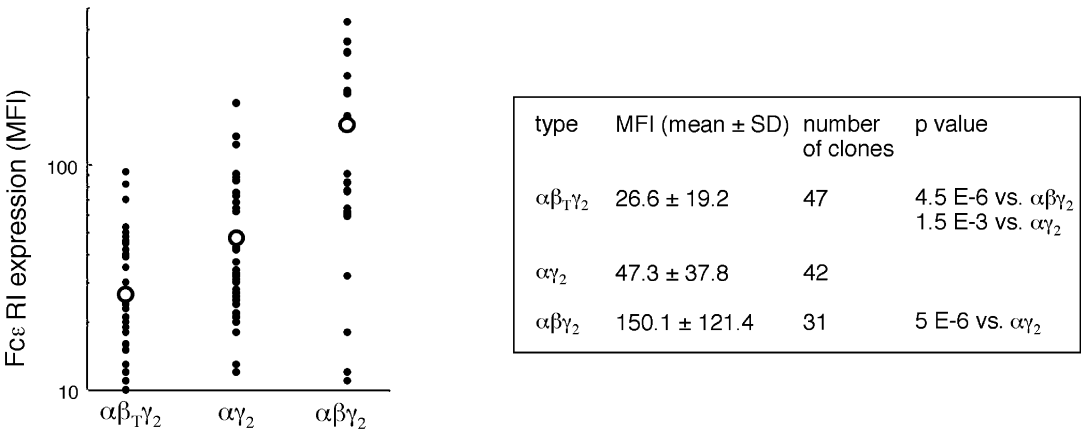


Figure 2. β_T Prevents Cell Surface Expression of Fc ϵ RI
Fc ϵ RI expression was assessed by flow cytometry after staining with IgE in $\alpha\beta_T\gamma_2$, $\alpha\gamma_2$, and $\alpha\beta\gamma_2$ transfectants and is expressed as mean fluorescence intensity (MFI). The means $\pm$ SD of MFI for all the clones obtained in each transfection type were calculated and compared with the Student's unpaired t test (three independent sets of transfections).

maturation was investigated. Three clones each of the $\alpha\gamma_2$, $\alpha\beta\gamma_2$, and $\alpha\beta_T\gamma_2$ transfectants were selected for their low, medium, and high levels of Fc ϵ RI expression. The α chain was immunoprecipitated and blotted. As expected, β promotes the maturation of α chain, as there was less immature α in the $\alpha\beta\gamma_2$ clones relative to the $\alpha\gamma_2$ clones with comparable amounts of mature α (Figure 3A) (Donnadieu et al., 2000b). With regard to β_T , immature α was substantially more abundant in the presence of β_T than in the absence of β for the same amount of mature α (Figure 3A, compare lanes 8 and 4 and lanes 9 and 5). The intensity of the mature α band was plotted as a function of the intensity of the immature α band for each clone (Figure 3B). The $\alpha\beta_T\gamma_2$ clones required much more immature α chain to express the same level of mature α chain than did the $\alpha\beta\gamma_2$ clones and the $\alpha\gamma_2$ clones. This indicates that α maturation is prevented when β_T is present and suggests that the inhibition of α chain maturation ultimately reduces surface expression of Fc ϵ RI.

β_T Has a Short Half-Life due to Rapid Targeting for Degradation
The opposite effects of β and β_T on Fc ϵ RI expression and α maturation led us to investigate the fate of the β_T

protein. Pulse-chase analysis was employed to compare the half-lives of β and β_T . One $\alpha\beta\gamma_2$ clone and two $\alpha\beta_T\gamma_2$ clones were labeled for 10 min and then transferred to unlabeled medium for a chase of 0 to 30 min. β and β_T were immunoprecipitated from their respective lysates and resolved by SDS-PAGE. Autoradiography followed by quantification of the β and β_T bands revealed that whereas β is stable over the 30 min chase, β_T has the remarkably short half-life of approximately 10 min (Figure 4A). The short half-life of β_T may reflect rapid targeting to a degradation pathway, such as the proteasome complex (Ciechanover, 1998; Cresswell and Hughes, 1997; Ward et al., 1995; Wiertz et al., 1996; Yang et al., 1998). The contribution of the proteasome to β_T degradation was assessed by the utilization of proteasome inhibitors, such as N-Acetyl-Leucyl-Leucyl-Norleucinal (ALLN), lactacystin, and carboxybenzyl-leucyl-leucyl vinylsulfone ZL₃VS (Wiertz et al., 1996). Pretreatment of β_T expressing cells with these inhibitors significantly increased the visibility of β_T following immunoprecipitation and immunoblot (Figure 4B). This suggests that the proteasome is one major mechanism by which β_T is degraded. Other degradation pathways may be involved as well, particularly since the specificity of these inhibitors for the proteasome is not absolute. Note

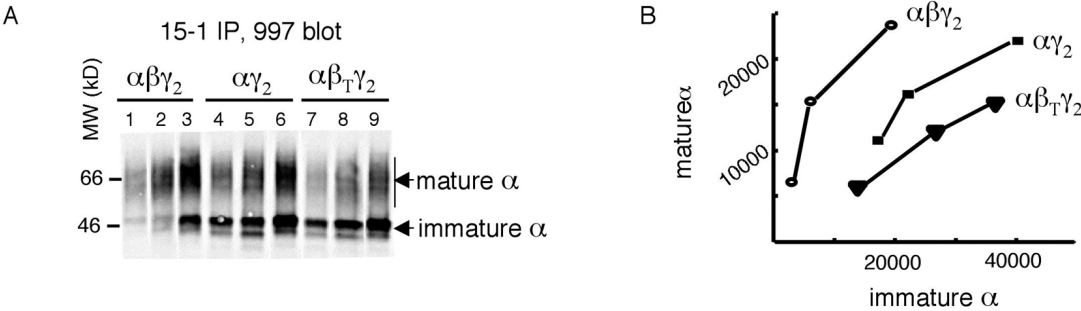


Figure 3. β_T Prevents Maturation of the α Chain
The α chain was immunoprecipitated from $\alpha\gamma_2$, $\alpha\beta\gamma_2$, and $\alpha\beta_T\gamma_2$ transfectants (three clones of each type) with the anti- α monoclonal antibody 15-1 that recognizes both the immature and mature forms of α . The immunoprecipitates were resolved by SDS-PAGE and immunoblotted with the polyclonal anti- α antibody 997 that recognizes all forms of α (A) (15 E6 cells/lane). The intensity of the immature and mature α bands was quantified by scanning. For each transfectant type, the amount of mature α was plotted as a function of the amount of immature α in each clone (B).

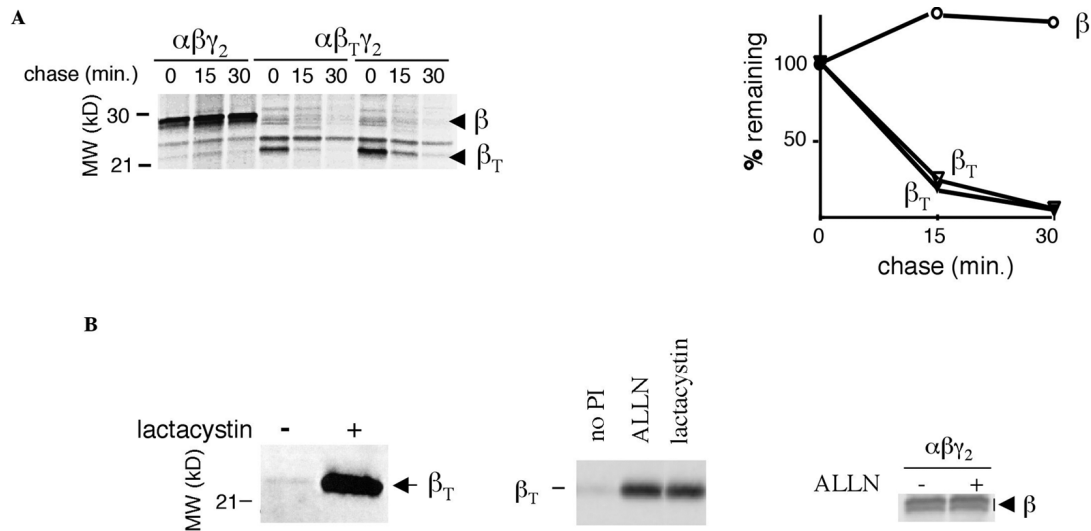


Figure 4. β_T Has a Very Short Half-Life Compared to β and Is Degraded by the Proteasome

(A) $\alpha\beta\gamma_2$ (one clone) and $\alpha\beta_T\gamma_2$ (two clones) transfectants were pulsed for 10 min and chased for 0–30 min. β and β_T were immunoprecipitated from labeled lysates with an anti-FLAG antibody and resolved by SDS-PAGE (left panel) (20 E6 cells per time point). Radioactivity associated with the β - and β_T -specific bands was quantified by scanning and plotted as a function of time (right panel). Note that the radioactivity levels measured here are well within the linear range (1–100,000) of the Storm system. The highest count detected in the analysis was 23,000. (B) $\alpha\beta_T\gamma_2$ transfectants were incubated with the proteasome inhibitor lactacystin (+) or with vehicle (–), and the β_T protein was immunoprecipitated and blotted (15 E6 cells/lane). β_T is easily detected after pretreatment with lactacystin, but was barely visible in untreated cells. The proteasome inhibitors ALLN and lactacystin have comparable effects on β_T (5 E6 cells/lane). ALLN minimally affects the β detection level.

that in the results presented in Figure 1D, the cells were incubated with a proteasome inhibitor prior to lysis. The expression level of β protein, on the other hand, is not affected by treatment with proteasome inhibitor (Figure 4B). This is in line with the stability of β seen during pulse-chase labeling (Figure 4A). These results indicate that the short half-life and degradation of β_T are specific to this particular product of the β gene.

β_T Associates with Immature α Chain and Not with Mature α

The results presented above indicate that β_T acts as a negative regulator of *FcεRI* surface expression, inhibits α maturation, and is rapidly degraded. These data provide a model for the mechanism of β_T function in which β_T would inhibit α maturation by associating with immature α in the ER and preventing immature α from reaching the Golgi. According to this model, β_T would be associated with immature α but not with mature α . The association of β_T with α was thus analyzed. Cell lysates were prepared from $\alpha\beta\gamma_2$ and $\alpha\beta_T\gamma_2$ transfectants and from untransfected cells. Half of each lysate was treated with Endo H to distinguish Endo H-resistant mature α from Endo H-sensitive immature α . All of the lysate fractions were then immunoprecipitated with anti-FLAG antibody (to immunoprecipitate β and β_T) and immunoblotted with the polyclonal anti- α (997) that recognizes all α forms. As expected, given the capacity of β to promote α maturation, the $\alpha\beta\gamma_2$ transfectants displayed very little, if any, association between β and immature α . There was a substantial amount of interaction, however, between β and mature α (Figure 5A, lane 4). This result was not altered by Endo H treatment, which is consistent with the fact that mature α is not Endo H sensitive (Figure 5A, compare lanes 3 and 4). Note that association of β

with immature α must occur during processing of the receptor. However, it is likely that the strong action of β as an amplifier of α chain maturation makes this step a rather transient state. As a result, β -associated immature α represents a small fraction of the α pool in a static analysis such as standard immunoprecipitation. The situation is the opposite in the $\alpha\beta_T\gamma_2$ transfectants (Figure 5A, lanes 5 and 6). The main form of α associated with β_T was immature α , as mature α was not detected after β_T precipitation (Figure 5A, lane 6; the 50 kDa band that is present also in the untransfected cells is the heavy chain of the precipitating Ab). The immature α band associated with β_T was Endo H sensitive, confirming that β_T -associated α was not terminally glycosylated in the Golgi (Figure 5A, compare lanes 5 and 6). These results support the model wherein the effect of β_T on α maturation is direct and is mediated by association of β_T with immature α in the ER. They suggest the existence of two pools of immature α in β_T -containing cells. One pool is not associated with β_T but does interact with a dimer of γ chains. This pool undergoes Golgi maturation and is expressed at the cell surface as $\alpha\gamma_2$ complexes. The other pool of immature α is associated with β_T . It does not undergo maturation and most likely does not reach the surface. The fate of this pool was further investigated.

β_T Does Not Associate with Properly Folded α Chain

In addition to the glycosylation maturation described above, the immature α chain undergoes folding in the ER, which creates a secondary structure capable of binding IgE. Proper folding requires mannose-rich glycosylation of α (Letourneur et al., 1995). Properly folded α and misfolded α can be distinguished based on their reactivity with various anti- α antibodies. The monoclonal

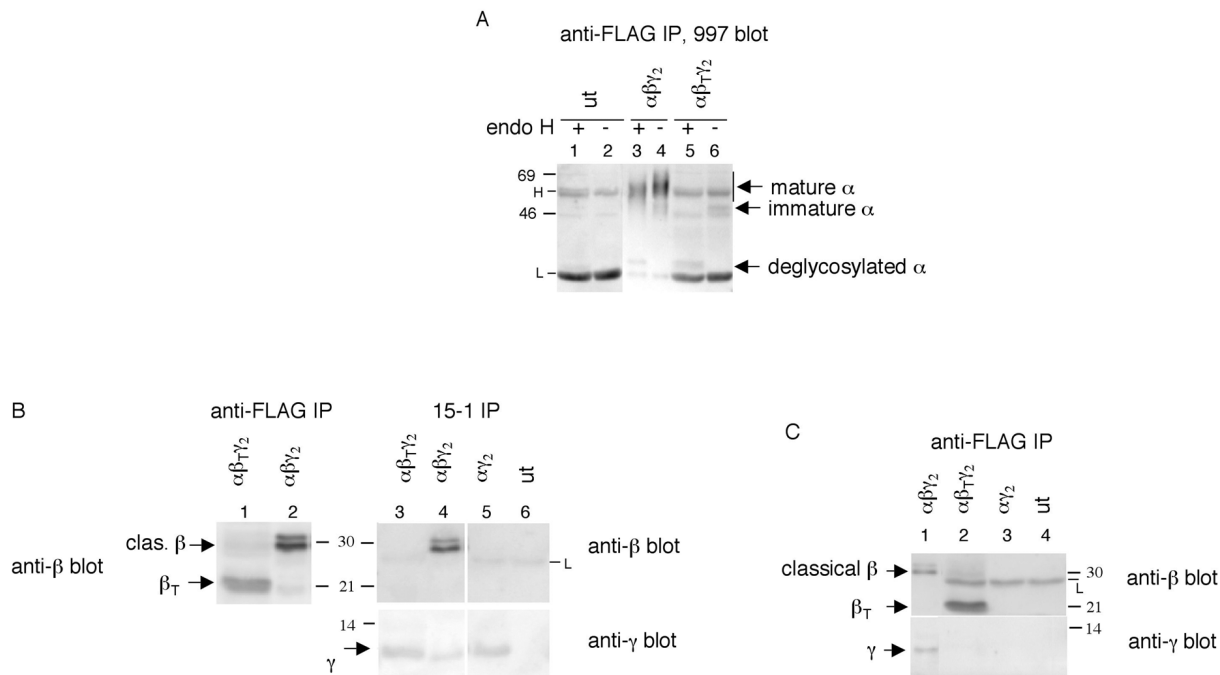


Figure 5. β_T Is Not Associated with Mature, Properly Folded α or with γ

(A) Untransfected U937 (ut), $\alpha\beta_T\gamma_2$, and $\alpha\beta\gamma_2$ transfectants were incubated with proteasome inhibitor and lysed. Half of each lysate was treated with Endo H. All of the lysate fractions were precipitated with anti-FLAG and blotted with polyclonal anti- α 997. Please note that in this experiment, cells numbers were adjusted so that comparable amounts of Endo H deglycosylated α would be present in the $\alpha\beta\gamma_2$ and $\alpha\beta_T\gamma_2$ samples (untransfected, $\alpha\beta_T\gamma_2$, 40 E6 cells/lane; $\alpha\beta\gamma_2$, 1.5 E6 cells/lane).

(B) Untransfected U937 (ut), $\alpha\beta_T\gamma_2$, $\alpha\beta\gamma_2$, and $\alpha\gamma_2$ transfectants were incubated with proteasome inhibitor and lysed under mild conditions (Triton X-100 0.25%). Half of each cell lysate was precleared with normal mouse IgG and immunoprecipitated with 15-1, a monoclonal antibody that recognizes only properly folded α (lanes 3–6). Immunoprecipitates were separated by Western blot and blotted. The blot was incubated with polyclonal anti- β 961 and anti- γ 934 Abs (lanes 3–6). As a control for the presence of β and β_T , the remaining half of the lysates was immunoprecipitated with anti-FLAG Ab and immunoblotted with anti- β (lanes 1 and 2). Please note that in this experiment, cell numbers were adjusted so that more γ would be present in the $\alpha\beta_T\gamma_2$ immunoprecipitate than in the $\alpha\beta\gamma_2$ immunoprecipitate (untransfected, $\alpha\gamma_2$, $\alpha\beta_T\gamma_2$, 20 E6 cells/lane; $\alpha\beta\gamma_2$, 2.5 E6 cells/lane).

(C) Untransfected U937 (ut), $\alpha\beta_T\gamma_2$, $\alpha\beta\gamma_2$, and $\alpha\gamma_2$ transfectants were processed as in (B). The lysates were immunoprecipitated with anti-FLAG Ab and immunoblotted with anti- β and anti- γ 934. Note that in this experiment, the minimum amount of $\alpha\beta\gamma_2$ lysate that preserves detection of associated γ was used. The amount of $\alpha\beta_T\gamma_2$ lysate, however, was adjusted such that more β_T would be present in the $\alpha\beta_T\gamma_2$ immunoprecipitate than β would be in the $\alpha\beta\gamma_2$ immunoprecipitate (untransfected, $\alpha\gamma_2$, $\alpha\beta_T\gamma_2$, 20 E6 cells/lane; $\alpha\beta\gamma_2$, 0.5 E6 cells/lane).

Ab 15-1 recognizes only properly folded α , whereas the anti-peptide polyclonal antibody 997 recognizes all forms of α (Letourneur et al., 1995; Wang et al., 1992). As expected, when lysate from $\alpha\beta\gamma_2$ transfectants was immunoprecipitated with the anti- α Ab (15-1), β and γ were coprecipitated (Figure 5B, lane 4). β_T , however, did not coprecipitate with properly folded α chain in $\alpha\beta_T\gamma_2$ transfectants (Figure 5B, lane 3). Expression of the β_T protein was verified via precipitation with anti-FLAG (Figure 5B, lane 1). The possibility that the failure to coprecipitate β_T with properly folded α was due to dissociation during immunoprecipitation was ruled out since γ was coprecipitated with α in $\alpha\beta_T\gamma_2$ transfectants. Note that in this experiment, cell numbers were adjusted so that more γ would be present in the $\alpha\beta_T\gamma_2$ immunoprecipitate than in the $\alpha\beta\gamma_2$ immunoprecipitate. This ensures that if complexes between mature α , β_T , and γ_2 were present, they would be abundant enough to be detected. In spite of the fact that more γ was coprecipitated in $\alpha\beta_T\gamma_2$ transfectants than in $\alpha\beta\gamma_2$ transfectants, β_T was not detected in a complex with properly folded α whereas β was (Figure 5B, compare lanes 3 and 4). This result

shows that β_T does not associate significantly with properly folded α and implies that the pool of immature α that is associated with β_T is not properly folded.

β_T Does Not Associate with the γ Chain

The association between β_T and γ was likewise investigated. $\alpha\beta_T\gamma_2$, $\alpha\beta\gamma_2$, and $\alpha\gamma_2$ transfectants were immunoprecipitated with anti-FLAG antibody and blotted with either anti- β or anti- γ antibody. The anti- β blot confirmed the presence of β and β_T in the $\alpha\beta\gamma_2$ and $\alpha\beta_T\gamma_2$ clones, respectively. The anti- γ blot indicated that association could be easily detected between β and γ , but not between β_T and γ , in spite of the fact that the amount of $\alpha\beta\gamma_2$ lysate was purposely reduced relative to that of $\alpha\beta_T\gamma_2$ lysate (Figure 5C, compare lanes 1 and 2). This suggests that β_T does not associate with γ to a significant extent and that, consequently, the pool of immature α associated with β_T is not associated with γ , further preventing its expression at the cell surface.

The sum of the analyses presented here provides strong biochemical evidence that emphasizes the contrasting functions of β and β_T and supports our model

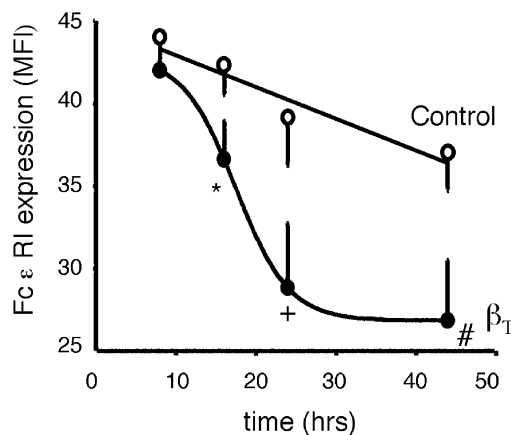


Figure 6. β_T Can Compete with β

A U937 clone stably transfected with $\alpha\beta\gamma_2$ was transiently retransfected with β_T cDNA or a control cDNA, and with a GFP construct. FcεRI expression at the cell surface was measured by flow cytometry (as in Figure 2) over time after gating on the GFP-positive population. FcεRI expression after β_T transfection was compared to expression after control transfection at the same time point using the Student's unpaired t test. * $p < 0.03$, + $p < 0.006$, # $p < 0.005$.

for the mechanism of β_T function. Specifically, unlike β , which is a robust amplifier of α chain processing and is only found to detectably associate with γ and mature α , β_T is only found to associate with immature and misfolded α . β_T neither associates with γ nor with mature and properly folded α . Based on the evidence that β_T is not associated with Endo H-resistant α , we can assume that this complex never makes it through terminal glycosylation in the Golgi. This is highlighted by the fact that β_T does not associate with γ chain, which is a prerequisite for receptor expression at the cell surface (Kinet et al., 1987, 1988; Blank et al., 1989) and by the demonstration that mature and properly folded α is the only form of α found at the plasma membrane (Letourneur et al., 1995). These data support a model that is in line with the short half-life of β_T and with the reduction in FcεRI surface expression that is associated with β_T expression. This model dictates that β_T associates with α early in the ER, prevents its maturation, and sequesters it from potential interaction with β and γ , and that β_T is degraded by itself or in complex with α before reaching the cell surface.

β_T Competes with β to Control FcεRI Surface Expression

Thus far, all the β expressing cell lines and primary cells that have been tested have been found to simultaneously coexpress β and β_T (see Figures 1A and 1C). This coexpression raises the exciting possibility that β_T is capable of competing with β to modulate FcεRI expression levels at the cell surface. Consequently, if β_T can compete with β , β_T should be able to decrease FcεRI expression in stable $\alpha\beta\gamma_2$ transfectants. To test this hypothesis, $\alpha\beta\gamma_2$ clones expressing constant levels of FcεRI at the cell surface were transiently transfected with β_T cDNA or a control cDNA. FcεRI expression at the cell surface was followed over time. Transfection with β_T cDNA but not with control cDNA significantly decreased FcεRI expression (Figure 6). Moreover, the

receptor levels remained decreased for more than 24 hr in the presence of β_T (Figure 6). These results show that β_T is capable of competing with classical β and provide support for the concept that the relative amounts of β and β_T influence the level of FcεRI at the cell surface. It is important to note that the data presented in this Figure as well as in Figure 2 were yielded in the absence of proteasome inhibitors. Thus, even in conditions under which β_T protein is difficult to detect, the effect on FcεRI surface expression is dramatic.

Discussion

Based on evidence garnered from the human genome project and from human expressed sequence tag databases, it is presently estimated that the genome contains approximately 35,000 genes that code for close to 100,000 proteins (Consortium, 2001; Venter, 2001). The disparity between these values highlights the importance of proteome expansion in eukaryotic cells. Among protein diversification mechanisms, alternative splicing is gaining increasing recognition as an important means by which to produce several proteins from one gene. Current predictions suggest that 35%–59% of human genes are variably spliced (Modrek and Lee, 2002; Sorek and Amitai, 2001), thus positioning alternative splicing as the predominant mechanism of proteome expansion in vertebrates (Modrek and Lee, 2002; Tasic et al., 2002). Here, we provide evidence that alternative splicing of the *FcεRIβ* gene results in two β isoforms that have antagonistic functions. Whereas the classical β form functions as an amplifier of FcεRI receptor signaling and expression, the splice variant β_T is an inhibitor of receptor expression.

The β gene has been shown to be one of the genetic factors that contribute to allergic phenotypes. Preliminary evidence suggests that in individuals, β and β_T are coexpressed. As indicated herein, β_T can compete with β and oppose its amplifier effect. This raises the possibility that regulated production of these two isoforms gauges cell-surface levels of FcεRI, not only in transfected cells but also in normal mast cells and basophils. Preferential expression of β over β_T would result in increased expression of the tetrameric form of FcεRI, $\alpha\beta\gamma_2$, along with its strong signaling capacity, thus contributing to the creation of an atopic phenotype. Conversely, preferential expression of β_T would result in a low expression level of the poorly signaling $\alpha\gamma_2$. The relative role in regulating FcεRI expression played by the β/β_T pair and by other factors, such as serum IgE levels, remains to be determined.

The factors that regulate splicing of the β gene are not known. Numerous factors have been reported to affect splicing in general (Lopez, 1998; Nissim-Rafinia and Kerem, 2002; Weg-Remers et al., 2001). Among those, some are attractive candidates for β splicing regulation, such as polymorphisms located near splice sites and phosphorylation of regulatory molecules in response to environmental stimuli.

Tools to manipulate gene expression and pre-mRNA processing are presently being developed, such as antisense oligonucleotides and RNAi (Cullen, 2002; Opalinska and Gewirtz, 2002). It is thus possible to envision

that the amount of β_T relative to β may be deliberately altered. The production and delivery of oligonucleotides that bind to the intron 5 splice sites would render them undetectable to the spliceosome. The presumable result of such interference would be an increase in the relative amount of β_T and a consequent decrease in Fc ϵ RI surface expression. In a therapeutic model, one would predict a concomitant decrease in susceptibility to allergic disorders. Given the impact of Fc ϵ RI expression on the pathophysiology of atopy, an understanding of the molecular mechanism by which β and β_T are produced will have profound implications on research in the area of allergic diseases.

Experimental Procedures

Cells

Semipurified basophils (2%–5% basophils) were obtained from blood donors by Percoll (Pharmacia) centrifugation (density 1.08) (Leonard et al., 1984). CBMC were prepared by growing cord blood mononuclear cells with 100 ng/ml recombinant human stem cell factor (Biosource, Camarillo, CA), 10 ng/ml recombinant human IL-6 (Endogen, Woburn, MA), and 1 μ M prostaglandin E₂ (Cayman Chemical, Ann Arbor, MI) for 10 weeks (Saito et al., 1995). The human basophilic cell line KU812, the human monocytoid cell line U937, and the mouse fibroblastic cell line NIH 3T3, untransfected and transfected, were maintained as described (Donnadieu et al., 2000b; Lin et al., 1996).

RNA Preparation

PolyA RNA was isolated from CBMC using Fast-Tack 2.1 (Invitrogen, Carlsbad, CA). Total RNA was extracted from all other cells using RNeasy B (Tel-Test, Inc, Friendswood, TX).

RT, PCR, and Cloning of PCR Products

Reverse transcription was performed using Omniscript reverse transcriptase (Qiagen, Valencia, CA) and oligodT. PCR was performed in 50 μ l final volume with 10 μ l RT reaction (equivalent to 0.1 μ g RNA), 1.25 U Taq polymerase (Fisher Scientific, Pittsburgh, PA), 200 μ M dNTPs, 60 pmol of forward (GTGCCTGCATTGAAGTCTTG) and reverse (TGGATCCTTGGCTGTGAATC) primers, 1.5 mM MgCl₂, 20 mM Tris (pH 8.3), and 25 mM KCl. Cycling was 94°C 45 s, 60°C 45 s, and 72°C 1 min for 25 cycles. PCR products were cloned by TA cloning in the pCRII vector (Invitrogen).

RNase Protection Assay (RPA)

RPA was performed using the RPAIII kit (Ambion, Austin, TX) with an antisense probe specific for human or mouse β generated with Maxiscript (Ambion). The human β probe covers the last 12 bases of exon 4, the entirety of exon 5, and the first 41 bases of intron 5 (Genbank accession number M89796) (Kuster et al., 1992). The mouse β probe covers the last 12 bases of exon 4, the entirety of exon 5, and the first 103 bases of intron 5 (Genbank accession numbers NM013516 and AB033617) (Ra et al., 1989). Ten micrograms of total RNA was used for U937 and C57 samples, whereas polyA RNA from 10E6 cells was used for CBMC samples. A Fc ϵ R γ probe was used as positive control for U937 samples and CBMC.

Generation of the Classical β and β_T Expression Constructs, and Transfections

An untagged β_T template was generated by PCR using β cDNA (Kuster et al., 1992) as template, a forward primer including the ATG, and a reverse primer including the first 51 bases of intron 5 up to the in-frame stop codon (Kuster et al., 1992). This β_T cDNA, as well as β cDNA, were then tagged by addition of a FLAG sequence (DYKDDDDK) at the 5' end by PCR. The tagged β and β_T inserts were ligated independently into the mammalian expression vector pBJ1neo (Lin et al., 1990). Insert sequences were verified by DNA sequencing. U937 and NIH3T3 cells were cotransfected by electroporation with 10 μ g each of Fc ϵ RI α and Fc ϵ RI γ and 2 μ g each of either β , β_T , or the empty vector pBJ1 neo. Stable clones were

selected in G418. Transient transfection with the β_T construct, or an irrelevant construct, and a GFP construct in U937 and KU812 $\alpha\beta\gamma_2$ clones was performed as described (Donnadieu et al., 2000b).

Flow Cytometric Analysis of Fc ϵ RI Expression at the Cell Surface

Fc ϵ RI expression in stable and transient transfectants was analyzed after binding of biotinylated recombinant IgE (Schwarzbaum et al., 1989) and streptavidin-phycoerythrin (Donnadieu et al., 2000b). For transient transfection, Fc ϵ RI expression was measured after gating on GFP-positive cells.

Immunoprecipitation, Western Blotting, Proteasome Inhibitor Treatment, and Endo-H Treatment

Immunoprecipitation and Western blotting were performed as in Donnadieu et al. (2000b), except in Figure 5 where cells were lysed in Triton X-100 0.25%. The following antibodies (Ab) were used: anti-human Fc ϵ RI α monoclonal Ab 15-1 (Wang et al., 1992), anti-human Fc ϵ RI α rabbit polyclonal Ab 997 (Letourneur et al., 1995), anti-human β rabbit polyclonal Ab 961 (Maurer et al., 1996), and anti-FLAG M2 monoclonal Ab (Sigma, St. Louis, MO). In Figures 1D, 4B, and 5, cells were treated with one of the following proteasome inhibitors, ALLN (Calbiochem, San Diego, CA) 250 μ M, lactacystin (Calbiochem) 10 μ M, carboxybenzyl-leucyl-leucyl vinylsulfone (ZL₃VS) 20 μ M (Wiertz et al., 1996), or vehicle (DMSO) for 2 hr at 37°C before lysis. In Figure 5A, immunoprecipitates were treated with endo- β -N-acetylglucosamidase (Endo H) (New England Biolabs) as previously described (Letourneur et al., 1995).

Pulse-Chase Analysis

Cells (5×10^5 /ml) were pulsed with 0.2 mCi/ml TRAN ³⁵S-LABEL (ICN, Costa Mesa, CA) for 10 min at 37°C and chased for the indicated periods of time in cold medium. At each time point, 15×10^6 cells were lysed in 0.5% Triton X-100, 300 mM NaCl, and 50 mM Tris (pH 7.5) in the presence of protease inhibitors (Complete, Roche Molecular Biochemicals, Indianapolis, IN). Lysates were precleared and then immunoprecipitated with anti-FLAG antibody as above. Proteins were separated on SDS 14% polyacrylamide gels, and the dried gels were exposed to a PhosphorImager screen (Molecular Dynamic, Sunnyvale, CA). Radiolabeled proteins detected by scanning of the exposed screen were quantified with the ImageQuant software (Molecular Dynamics).

Statistics

Means are expressed $\pm$ SD. Comparisons between groups were performed with the Student's unpaired t test.

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