Signal Transduction by Immunoglobulin Is Mediated Through Igα and Igβ

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Summary

Immunoglobulin (Ig) antigen receptors are composed of a noncovalently-associated complex of Ig and two other proteins, Igα and Igβ. The cytoplasmic domain of both of these Ig associated proteins contains a consensus sequence that is shared with the signaling proteins of the T cell and Fc receptor. To test the idea that Igα-Igβ heterodimers are the signaling components of the Ig receptor, we have studied Ig mutations that interfere with signal transduction. We find that specific mutations in the transmembrane domain of Ig that inactivate Ca^2+ and phosphorylation responses also uncouple IgM from Igα-Igβ. These results define amino acid residues that are essential for the assembly of the Ig receptor. Further, receptor activity can be fully reconstituted in Ca^2+ flux and phosphorylation assays by fusing the cytoplasmic domain of Igα with the mutant Igs. In contrast, fusion of the cytoplasmic domain of Igβ to the inactive Ig reconstitutes only Ca^2+ responses. Thus, Igα and Igβ are both necessary and sufficient to mediate signal transduction by the Ig receptor in B cells. In addition, our results suggest that Igα and Igβ can activate different signaling pathways.

Membrane Ig plays a central role in the generation of immune responses. However, there has been limited progress towards understanding the molecular mechanism of action of this receptor. Two features of receptor Igs have made functional analysis a difficult problem. First, the intracytoplasmic domain of the receptor is composed of three amino acids that offer no specific clues about the mechanism of signaling. Second, Igs are associated with several other polypeptides on the cell surface to form a multi-subunit structure (1-4). Two of these receptor-associated polypeptides, Igα and Igβ, have been implicated in receptor assembly and cell surface transport (3, 5). In addition, both Igα and Igβ share a consensus sequence with signaling proteins of the T cell and Fc receptors (6). Crosslinking surface Ig leads to phosphorylation of Igα and Igβ and activation of receptor-associated kinases (4, 7-11). Indeed, functional Ig antigen receptors can be reconstituted in T cells by cotransfection of IgM, Igα, and Igβ and a direct physical link between the cytoplasmic domains of Igα and Igβ and intracellular kinases has been proposed (12, 13). Although these experiments suggest an important role for the Igα-Igβ complex in signal transduction, the key questions of how the Igs are linked to the associated proteins, and whether Igα and Igβ mediate signaling in B cells have not been resolved. To address these issues we have examined B cell lines that carry Igs with specific transmembrane mutations that impede signal transduction (14-17).

Materials and Methods

**DNA Constructs.** Unique BamHI, and NotI sites surrounding the membrane exons of human IgM were placed in p468, a construct that contains Ig H and L chain genes (13). The membrane exons were then subcloned, and 587-588 YS/VV, as well as an in-frame unique NcoI-XbaI linker were inserted at position 594 by site-directed mutagenesis (p509), Igα and Igβ cytoplasmic tails and mutant versions with NcoI and XbaI sites at the 5' and 3' ends were amplified from plasmids (10467 and p466, respectively [13]) by PCR. After sequence reification, the PCR fragments were subcloned in-frame at the end of the Ig transmembrane domain. The modified membrane exons were then reinserted into the Ig in expression vector (p468) by unique BamHI and NotI sites (p520 IgM:Igβ; p523 IgM:Igα; p519 IgM:Igβ-Y/F 206).

**Cell Lines.** A20 cells were grown in RPMI-1640 supplemented with 5% bovine calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM l-glutamine. Cells were transfected with linear plasmid DNA by electroporation (17a). Selection was carried out with 550 μg/ml G418 (Gibco BRL, Grand Island, NY). Transfected cells were enriched for surface Ig expression by staining with fluorescein goat anti-human IgM (Southern Biotechnology Associates, Birmingham, AL), and sorting on a FACStar Plus® (Becton Dickinson & Co., Mountain View, CA). Analysis of surface staining
was performed with either fluorescein goat anti-mouse IgG2a or biotinylated monoclonal anti-human IgM and fluorescein-streptavidin (Pharmingen, San Diego, CA) using a FACScan® (Becton Dickinson & Co.).

Abs. 5 mg of purified polyclonal goat anti-human IgM or goat anti-mouse IgG2a (Southern Biotechnology Associates) were preabsorbed on 1 mg of mouse IgG2a or human IgM, respectively coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ). The polyclonal Abs did not crossreact as measured by staining and immunoprecipitation. Monoclonal anti-human IgM (18) was purified and modified as previously described (13). Abs specific for Igα and Igβ were produced in rabbits by immunization with fusion proteins that combined the cytoplasmic domain of Igα or Igβ with glutathione S-transferase (19). Both sets of Abs immunoprecipitated a disulfide-linked heterodimer from B cells, and from T cells transfected with IgM, Igα, and Igβ. Neither of the two Abs reacted with extracts from untransfected T cells (data not shown). In immunoblots, the anti-Igα Ab revealed a single 30-Kd protein in B cell lysates or T cells transfected with Igα. The anti-Igβ Ab blotted a set of three 32-, 37-, and 39-Kd proteins from B cells, and Igβ transfected T cells. There was no crossreactivity between the anti-Igα and Igβ Abs.

Immunoprecipitation and Immunoblotting. For immunoprecipitation experiments cells were lysed in 1% n-dodecyl-B-D-maltoside (Anatrace, Maumee, OH) or 1% digitonin (Sigma Chemical Co., St. Louis, MO), 0.5 M Tris, pH 6.8, 100 mM NaCl. Insoluble material was removed by centrifugation and the supernatant fraction was incubated with either goat anti-IgG2a or anti-IgM coupled directly to cyanogen bromide-activated Sepharose (Pharmacia Fine Chemicals). Immune complexes were collected, washed in lysis buffer, separated by 9% reducing SDS-PAGE, and transferred to Immobilon-P (Millipore Co., Bedford, MA) in a semidry transfer system (Owl Scientific Plastics, Cambridge, MA). After incubation with blotting Abs, labeled proteins were visualized with 125I-protein A (New England Nuclear, Boston, MA), using either a Phosphorimager (Molecular Dynamics, Sunnyvale, CA), or x-ray film.

Induction of Tyrosine Phosphorylation. Transfected A20 cells (2 × 10^7/ml) were crosslinked at 37°C with 20 µg/ml of anti-IgG2a or 10 µg/ml monoclonal anti-human IgM (18) for the indicated times in minutes. The cells were lysed with 1% NP-40, 10 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 10 µg/ml aprotinin/leupeptin, 100 µM Na3VO4, pH 8, and the insoluble material was removed by centrifugation. Phosphotyrosine-containing proteins were immunoprecipitated with antiphosphotyrosine monoclonal PY20 (ICN Biochemicals, Cleveland, OH) and formalin-fixed Staphylococcus aureus (Pierce, Rockford, IL). Proteins were resolved on 9% SDS-PAGE, transferred to nitrocellulose, and immunoblots were performed with a rabbit polyclonal antiphosphotyrosine antiserum (19). Labeled proteins were visualized with 125I-protein A.

Ca2+ Flux Measurements. Cells loaded with Fura-2 (Molecular Probes Inc., Eugene, OR) were assayed spectrophotometrically for calcium mobilization (13) in response to 60 µg/ml of polyclonal anti-human IgM or 40 µg/ml of anti-IgG2a.

Figure 1. Association of Igα and Igβ is mediated through polar amino acids in the transmembrane domain of immunoglobulin. (A) Transmembrane and cytoplasmic domains of IgM, mutant Igs, and chimeric proteins. Identity with the wild-type protein is indicated with a dash, and amino acid differences are as shown. (B) Immunoprecipitation of Ig receptor complexes from transfected A20 cells. IgG2a and IgM receptor complexes were independently immunoprecipitated with Sepharose-coupled goat anti-mouse IgG2a or anti-human IgM. The blots were then probed with Abs to Igα, Igβ, or human IgM. (C) Comparison of dodecyl-maltoside and digitonin for coimmunoprecipitation of Ig receptor complexes. A20 cells were solubilized in either 1% dodecyl-maltoside or 1% digitonin, and immune complexes analyzed by SDS-PAGE followed by blotting with anti-IgM. (WT) Wild-type human IgM; (cyto-A) deletion of KVK cytoplasmic amino acids; (YS/VV) replacement of both tyrosine 587, and serine 588 with valine in the Ig transmembrane domain; (IgM:M1gα) fusion protein composed of nucleotides encoding amino acids 160-220 of Igα with the extracellular and transmembrane domain of YS/VV IgM; (IgM:Fabβ) fusion protein composed of nucleotides encoding amino acids 181-228 of Igβ with the extracellular and transmembrane domain of YS/VV IgM; (α) immunoprecipitation with anti-IgG2a; (α) immunoprecipitation with anti-IgM; (Igα) anti-Igα immunoblot; (Igβ) anti-Igβ immunoblot; (IgM) anti-IgM immunoblot; (DM) immunoprecipitation in 1% dodecyl-maltoside; (DI) immunoprecipitation in 1% digitonin.
port of IgM to the cell surface without altering membrane anchorage (14-17, 20). For example, introduction of non-polar groups such as val-val in place of Tyr-Ser at position 587-588 (YS/VV) produces a receptor that can no longer produce a calcium flux or present antigen (15). Similar changes also result in an Ig whose transport to the cell surface is independent of coexpression of Igα and Igβ (14). One explanation for these observations is that the polar amino acids in the Ig transmembrane domain bridge IgM and the Igα- Igβ heterodimer.

We used Abs to the cytoplasmic domains of Igα and Igβ to determine whether specific Ig transmembrane mutations that interfere with signal transduction also disrupt the association of IgM with Igα- Igβ. Endogenous and transfected Igs were immuno precipitated with specific anti-IgM or anti-IgG2a Abs, and the associated proteins were detected by Western blotting with anti-Igα or anti-Igβ. Endogenous IgG2a expressed by the A20 cells was always associated with both Igα and Igβ. When A20 cells were transfected with wild-type IgM, Igα and Igβ were coinmunoprecipitated with both the transfected IgM and endogenous IgG2a (Fig. 1B). In contrast, the cyto: Δ and YS/VV mutant IgM H chains that neither present antigen nor signal were not associated with the Igα- Igβ heterodimer. Neither Igα nor Igβ was coimmunoprecipitated with either of the two mutant Igs despite high level expression on the surface of transfected A20 cells (15) (Fig. 1B). Since both the cyto:Δ and the YS/VV H chain proteins have a normal extracellular domain, expression of this domain is not sufficient to mediate the interaction between IgM and Igα- Igβ. This observation may explain why synthesis of the secreted form of IgM does not interfere with the assembly of membrane Ig antigen receptors.

Our results, and those of other investigators (3, 14) suggested an important role for the transmembrane or cytoplasmic domains in stabilizing the IgM/Igα- Igβ receptor complex (3, 14). Both the cyto: Δ and YS/VV mutations specifically alter the transmembrane domain. The cyto: Δ mutant does so by producing an Ig protein that is anchored to the plasma membrane by an inositol linkage (20). Finding that this mutation destroys the normal noncovalent interactions between IgM and Igα- Igβ implied that transmembrane domain is required for this interaction. The YS/VV mutation pinpoints essential amino acids since altering these two polar residues destabilizes the IgM/Igα- Igβ complex, without changing Ig membrane anchorage (15, 20). The polar groups in the Ig transmembrane domain may interact with like residues in the transmembrane domains of Igα and Igβ to stabilize the IgM/Igα- Igβ receptor complex. This type of interaction is similar to that described for the α and β chains of the TCR (21, 22), and appears to be another common theme in the family of immune recognition receptors (23). The IgM we used was human and the Igα and Igβ were mouse, but the key amino acids are completely conserved in IgM between species (24). This human IgM is functional in transgenic mice for allelic exclusion and induction of B cell development, and can trigger normal proliferative responses when crosslinked in mature B cells (25, 26). Further, similar mutations in mouse IgM have effects on receptor transport that were consistent with uncoupling of Ig from Igα and Igβ (14). Other combinations of human and mouse receptor components may be less favorable. For instance, the combination of human Igα with mouse Igβ leads to inefficient transport of mouse IgM to the cell surface (3). However the degree of conservation between human and mouse Igα is only 68% overall, and 56% in the extracellular domain which is required for intrachain disulfide bond formation between Igα and Igβ (27). We conclude that the polar groups in the Ig transmembrane domain are essential contact points for Igα- Igβ. In addition there is a strong correlation between receptor activity and the ability of the immunoglobulin heavy chain to form a stable complex with Igα- Igβ.

**Ig Transmembrane Mutations Interfere with Activation of Tyrosine Phosphorylation.** To further explore the functional significance of the association of Igα- Igβ with Ig, we examined the effects of the cyto:Δ and YS/VV mutations on the induction of phosphorylation by receptor crosslinking. A20 cell lines transfected with the mutant Igs were stimulated with anti-IgM or anti-IgG2a, and phosphorylation of cellular substrates was assayed in immunoblotting experiments with antiphosphotyrosine Abs. In control cell lines, crosslinking of membrane IgG2a induced a rapid increase in tyrosine phosphorylation, and in A20 cells transfected with wild-type IgM this response was elicited by crosslinking either IgM or IgG2a (Fig. 2). In contrast, the cyto:Δ mutation, which creates an inositol-anchored form of IgM, completely destroys the ability of the antigen receptor to stimulate tyrosine phosphorylation. The YS/VV mutation, which also uncouples the receptor from Igα- Igβ, dramatically reduces the induction phosphorylation but does not completely eliminate this response. This small amount of phosphorylation is reproducible, and not a function of crossreactivity since there is no response in cyto: Δ mutant or untransfected A20 cells (Fig. 2). One explanation for the low level activation by the mutant receptor is that the active conformation of Ig that is induced by crosslinking interacts weakly with Igα- Igβ. Diminution of the response in YS/VV may reflect the difference between a physical linkage of the components in the wild-type receptor, and a diffusion limited process in the mutant.

**The Cytoplasmic Domains of Igα and Igβ Are Sufficient to Reconstitute Signaling.** The finding that Igs that fail to signal also fail to associate with Igα- Igβ is consistent with the hypothesis that the Igα- Igβ heterodimer mediates signal transduction (6). To test this notion directly we produced chimeric receptors composed of the extracellular and transmembrane domains of YS/VV mutant Igs fused with the cytoplasmic domains of either Igα or Igβ. The mutant Igs were attractive candidates for reconstitution experiments since they do not interact with endogenous Igα- Igβ (Fig. 1B), but contain sequences that could be important for interaction with other as yet unidentified cellular factors. Chimeric Ig proteins were transfected into the A20 B cell line, and high level expression was obtained by cell sorting (Fig. 3B). We found that the chimeras did not associate with endogenous Igα- Igβ (data not shown), nevertheless the recombinant proteins
were able to reconstitute Ca$^{2+}$ responses (Fig. 3 C). Crosslinking the transfected IgM:Igε or IgM:Igβ chimeras with anti-IgM results in a Ca$^{2+}$ flux, whereas the parental YS/VV Ig was inactive (15, Fig. 3 C). The consensus sequence shared by Igα, Igβ, TCR, and FcR components includes tyrosines that have been shown to be essential for function (28). To examine the role of the conserved tyrosines, Tyr 206 in Igβ was changed to Phe in the IgM:Igε chimera (IgM:Igε-Y/F). This single change was sufficient to destroy the activity of the IgM:Igβ in Ca$^{2+}$ flux experiments, but did not alter Ca$^{2+}$ responses induced by crosslinking endogenous membrane IgG2a (Fig. 3, C and D). Thus the Ca$^{2+}$ response is dependent upon the presence of the conserved tyrosine at position 206 of Igβ. When the same cell lines were tested for induction of phosphorylation by receptor crosslinking, we found that only the Igε chimera was active (Fig. 3 E). Crosslinking the IgM:Igε chimera induced rapid tyrosine phosphorylation of a number of cellular proteins, whereas IgM:Igβ chimera did not give an appreciable response above the background (Fig. 3 E). Thus, both Igα and Igβ cytoplasmic domains were active in transferring signaling activity to YS/VV Ig, but there is a difference in their range of activity. Both stimulated Ca$^{2+}$ flux but only Igε was able to induce the rapid phosphorylation of a large number of cellular substrates. Further, the signaling mechanism for Ca$^{2+}$ flux is dependent on tyrosine 206 in Igβ which is part of a consensus sequence shared with other immune recognition receptors. The role of the conserved tyrosines in this consensus sequence has not been clearly elucidated. However, the corresponding residues in the TCR-ε chain are rapidly phosphorylated upon receptor activation, and only the phosphorylated forms of ε are associated with the zap-70 kinase (29, 30). Components of the Ig receptor have been associated with a number of different kinases including Lyn, Fyn, Blk, and Syk which is highly homologous to Zap-70 (7, 8, 29, 31). In B cells, Igα and Igβ may serve to connect IgM with cellular kinases by binding directly to SH2 domains. Elegant structural studies of src SH2 domains have demonstrated that the interaction of SH2 with peptides is through phosphotyrosine and isoleucine binding pockets spaced by two amino acids (32-34). The consensus found in the immune recognition receptors includes at least two YYXXL/I repeats that would be predicted to interact with the SH2 domains of Src family kinases (32-35). Indeed the cytoplasmic tails of Igε and Igβ bind to two different sets of kinases in cellular extracts (12). These in vitro experiments suggested that Igα and Igβ may mediate different signaling functions through interactions with different kinases. A number of cellular and developmental events in B cells are regulated by membrane Ig (25, 36-39). These events could all be mediated through the Igα-Igβ heterodimer, or through individual receptor components interacting with different sets of kinases. Our finding that Igα and Igβ have different signaling activities, provides evidence that Igα and Igβ can mediate independent physiologic processes in vivo.

These studies were designed to elucidate the molecular requirements for Ig receptor function. A key aspect of the strategy we used was specific Ig transmembrane mutations that were known to interfere with signal transduction. We have shown that association of IgM with Igα-Igβ is required to produce a functional B cell antigen receptor. That signal transduction can be reconstituted with the cytoplasmic do-
Figure 3. The cytoplasmic tails of Igα and Igβ reconstitute Ca\(^{2+}\) and phosphorylation responses in YS/VV mutant Igs. (A) Diagrammatic representation of DNA constructs that encode IgM and chimeric Igs. (B) Flow cytometric analysis of surface expression of chimeric human Ig constructs (IgM:Igα p523, IgM:Igβ p520, IgM:Igβ Y206/F p519, Fig. 1 A) transfected into A20 B cell lines and stained with fluorescein-labeled anti-human IgM (Southern Biotechnology Associates). Unstained cells are the negative control in each case. (Top) Transfected constructs. (C) Ca\(^{2+}\) flux in response to anti-IgM in A20 cells transfected with chimeric Igs. (D) Ca\(^{2+}\) flux in response to control anti-IgG2a in the transfected A20 cells. (E) Induction of tyrosine phosphorylation by cross-linking with anti-IgM or anti-IgG2a Abs in A20 cells transfected with chimeric receptors. (IgM[Igα]) A20 cells transfected with chimeric Ig-Igα; (IgM[Igβ]) A20 cells transfected with chimeric Ig-Igβ; (IgM[Igβ Y/F]) A20 cells transfected with mutant chimeric IgM:Igβ in which Tyr 206 in Igβ has been changed to Phe; anti-IgM, cells crosslinked with anti-IgM Abs; anti-IgG2a, cells crosslinked with anti-IgG2a Abs.

The cytoplasmic tails of Igα and Igβ imply that these sequences are also sufficient to mediate signaling. However Igα and Igβ had different biological activities suggesting that each of these molecules may have unique functions in B cell development and activation. The chimeric receptors that we have described, composed of mutant Igs and individual signaling components, should provide a powerful new tool for the further analysis of the physiologic role of Igα and Igβ.

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Note added in proof: Kim et al. have published similar signaling experiments using chimeras composed of CD8 and the cytoplasmic domains of Igα and Igβ. Eur. J. Immunol. 1993. 23:911.

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