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Protein 4.1G binds to a unique motif within the FcγRI cytoplasmic tail

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Abstract

The C-terminal domain of protein 4.1G was identified to interact with the cytosolic tail of the high affinity IgG receptor, FcγRI, in yeast two-hybrid screens. Proteins of the 4.1 family have previously been found to mediate receptor/cytoskeleton interactions. In the study presented here, we show an alternatively spliced 4.1G product to be associated with increased FcγRI binding in yeast two-hybrid assays, and to be selectively enriched in most immune cells at the transcript level. In addition, a detailed analysis of the 4.1G 'docking site' within FcγRI is provided by examining FcγRI-CY-truncated and alanine-substituted mutants. These pointed to an FcγRI membrane-proximal core motif of HxxBxxxBB (H represents hydrophobic residues, B basic residues and x represents any residue), followed by hydrophobic and (potentially) negatively charged residues to be central for interaction with protein 4.1G.

Keywords: Fc receptor; Yeast two-hybrid; Molecular interaction; Mutagenesis; Domain mapping

1. Introduction

Fc receptors (FcR) mediate interactions between immune cells and immunoglobulins (Ig) (Daeron, 1997; Ravetch and Bolland, 2001). Multiple FcR exist as heterologous complexes of ligand-binding α-chains and promiscuous subunits that carry intracellular tyrosine-based activation motifs (ITAM). With the exception of class II FcγR members, the α-chains of multi-chain FcR bear no recognized signaling motifs in their cytoplasmic domains. However, recent studies demonstrated the cytoplasmic domain of FcγRI to be important for protein interactions that modulate receptor function (Beekman et al., 2004a,b).

FcγRI (CD64) represents a high affinity IgG receptor that is constitutively expressed by myeloid cells. The 72 kDa type I glycoprotein can effectively capture small immune complexes and facilitates presentation of antigens in the context of MHC class I and II (Barnes et al., 2002; Ioan-Facsinay et al., 2002; van Vugt et al., 1999; Wallace et al., 2001). The FcγRI cytosolic domain (FcγRI-CY) of approximately 65 residues interacts with cytoskeleton-associated proteins like periplakin and filamin A (filamin or ABP-280) (Beekman et al., 2004a; Ohta et al., 1991). The role of these proteins as scaffold for FcγRI signaling complexes is not well understood, but may be profound as truncation of FcγRI-CY prevented FcR γ-chain independent antigen presentation, IL-6 production and lead to altered kinetics of endocytosis and phagocytosis (Edberg et al., 1999; van Vugt et al., 1999).

Here we studied interactions between FcγRI-CY and splice-forms of protein 4.1G, which were identified to bind FcγRI-CY in yeast two-hybrid screens. Protein 4.1 family members have been shown to functionally modulate multiple receptors (Gascard and Mohandas, 2000; Hoover and Bryant, 2000; Takakuwa, 2000). The C-terminus of protein 4.1G interacted with FcγRI-CY and was found selectively spliced in immune cells at the transcript level. Truncated and alanine-substituted mutant FcγRI-CY were assessed for interaction with 'immuno-enriched' 4.1G.

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2. Material and methods

2.1. DNA constructs, antibodies and chemicals

cDNA encoding the cytosolic tail of human FcγRI (and mutants), FcγRIIa, FcγRIIIa, FcαRI, FcεRI and murine (m) FcγRI were expressed from pGBT9 (Beekman et al., 2004a,b). Recombinant proteins were expressed from pGEX-2T (Amersham Biosciences, Amersham, UK) containing the original GST tag in case of FcγRI or containing a replaced His-tag (6× histidine) in case of 4.1G-CTDΔ14 (starts at bp 2121–2402 coupled to 2652–3062, GenBank accession number NM_001431) (Beekman et al., 2004b). Integrity of cDNA generated by PCR was verified by sequence analysis using BigDye Terminator mix (Applied Biosystems, Warrington, United Kingdom). Western blots were performed with anti-GST goat polyclonal serum (Amersham), rabbit serum against FcγRI-CY (a kind gift of Dr. S. Tridandapani, Department of Internal Medicine, Ohio State University, Columbus, OH, USA) and monoclonal antibody against 6× His-Gly (Invitrogen, Leek, The Netherlands). Detection was performed by using horse radish peroxidase-conjugated mAb (Pierce, Rockford, IL, USA and Dako, Glostrup, Denmark). Chemicals were from Sigma (Steinheim, Germany) unless indicated otherwise.

2.2. Yeast two-hybrid

A human bone marrow cDNA library in pACT-2 was screened for FcγRI-CY-interacting proteins in yeast strain YGH1 according to Clontech protocols (Clontech, Palo Alto, CA, USA). Protein interactions were assessed by growth of colonies on histidine depleted media and β-galactosidase activity using replica filter assays as described in Beekman et al. (2004a). Quantification of FcγRI interaction with protein 4.1G cDNA clones in yeast cells were performed in a liquid β-galactosidase assay using ONPG as substrate (protocol described in Clontech's Yeast Protocols Handbook).

2.3. RT-PCR

Transcripts of 4.1G were amplified from oligo-dT primed cDNA by 30 cycles (30 s 95 °C, 30 s 62 °C and 30 s 72 °C) using forward primers (Isogen, Maarssen, The Netherlands) that recognized a 4.1G-specific region in the first part of exon 14 (5′-accaccgagtgaccgagggc-3′) and reversed primers that hybridized adjacent to 4.1G stopcodon (5′-cagatgtgatggtttgtgcgg-3′). Monocytes were cultured towards immature dendritic cells (DC, day 5) and were matured for 2 days as described (Jonuleit et al., 1997; Sallusto and Lanzavecchia, 1994). Interferon γ (IFN-γ) stimulations were overnight with 300 U/ml (IFN-γ1b, Boehringer Ingelheim, Biberach, Germany). The presence of cDNA in samples was verified by GAPDH PCR. AmpliTaq Gold and PCR reagents were from Perkin-Elmer (Nieuwerkerk a/d IJssel, The Netherlands). PCR products were extracted from agarose and sequenced.

2.4. Co-immunoprecipitation of recombinant proteins

GST-FcγRI and His-tagged 4.1G-CTDΔ14 were purified under non-denaturing conditions as described in Beekman et al. (2004a). Tosyl-activated M-280 Dynabeads (Dynal, Oslo, Norway) were covalently attached to anti-GST antibody according to the manufacturers' instructions. Beads were incubated overnight with ∼1 μg of GST-FcγRI or GST and ∼1 μg of His-4.1G in 500 μl RIPA buffer (150 mM NaCl, 100 mM Tris–HCl, pH 8.3, 0.1% SDS, 0.5% DOC, 1% Triton X-100). Beads were washed three times in RIPA, boiled in Laemmli sample buffer and analyzed by SDS-PAGE and Western blotting.

2.5. Sequence alignments

Sequences of 4.1G-CTD-interacting proteins were obtained from Entrez Nucleotide at http://www.ncbi.nlm.gov/. GenBank accession numbers: PTA-1 NM_006566, D2/D3 dopamine receptors NM_000795/NM_000796, A1 adenosine receptor AF042079, nuclear mitotic apparatus protein (NuMa; Z11583) and FK506-binding protein 13 (FKBP13, XM_215196). GluR-1/GluR-D AMPA receptors were found in the Swiss-Prot database, accession number P19493/P19490. Secondary structures were predicted with PHD (Rost, 1996), FASTA (Pearson, 1990), SSPro (Baldi et al., 1999), 3D-PSSM (Kelley et al., 2000) and JPRED (Cuff et al., 1998). Sequences were edited using BioEdit software available at http://www.mbio.ncsu.edu/BioEdit/bioedit.html.

3. Results

3.1. FcγRI and 4.1G interact in yeast two-hybrid screens

The cytosolic domain of FcγRI and the FcR γ-chain both trigger cellular functions (Edberg et al., 1999; van Vugt et al., 1999). Little is known, however, about protein effectors of the FcγRI cytosolic tail, albeit that recent studies indicate protein interactions with FcγRI-CY to modulate receptor function (Beekman et al., 2004a). We, therefore, searched for novel FcγRI-CY-interacting proteins by yeast two-hybrid screens.

The C-terminal domain (CTD) of 4.1G was found to interact with FcγRI-CY in two screens of human bone marrow cDNA. Three-out-of-six identified cDNA had different inserts and β-galactosidase assays are shown in Fig. 1A and B. One cDNA (IV.140) displayed reduced interaction with FcγRI as it showed no blue staining after filterlift and reduced β-galactosidase activity. Protein 4.1 members have three conserved regions (FERM, spectrin–actin-binding domain (SABD), CTD) that are each preceded by unique sequences (Fig. 1C). The topology of FcγRI-CY-interacting 4.1G cDNA is indicated in Fig. 1C. The smallest cDNA (IV.53) encompassed the complete C-terminal domain of 4.1G and started at the end of exon 14. Four cDNA (I.58, IV.32, IV.36, IV.58; or 4.1GΔ14) started at the beginning of exon 14, however, carried in-frame deletions of the second half of exon 14 which was cleaved at site caag↓guag (a conserved splice sequence is underlined). The largest cDNA (IV.140 or 4.1GΔSABD) started at bp 1782, but lacked exons

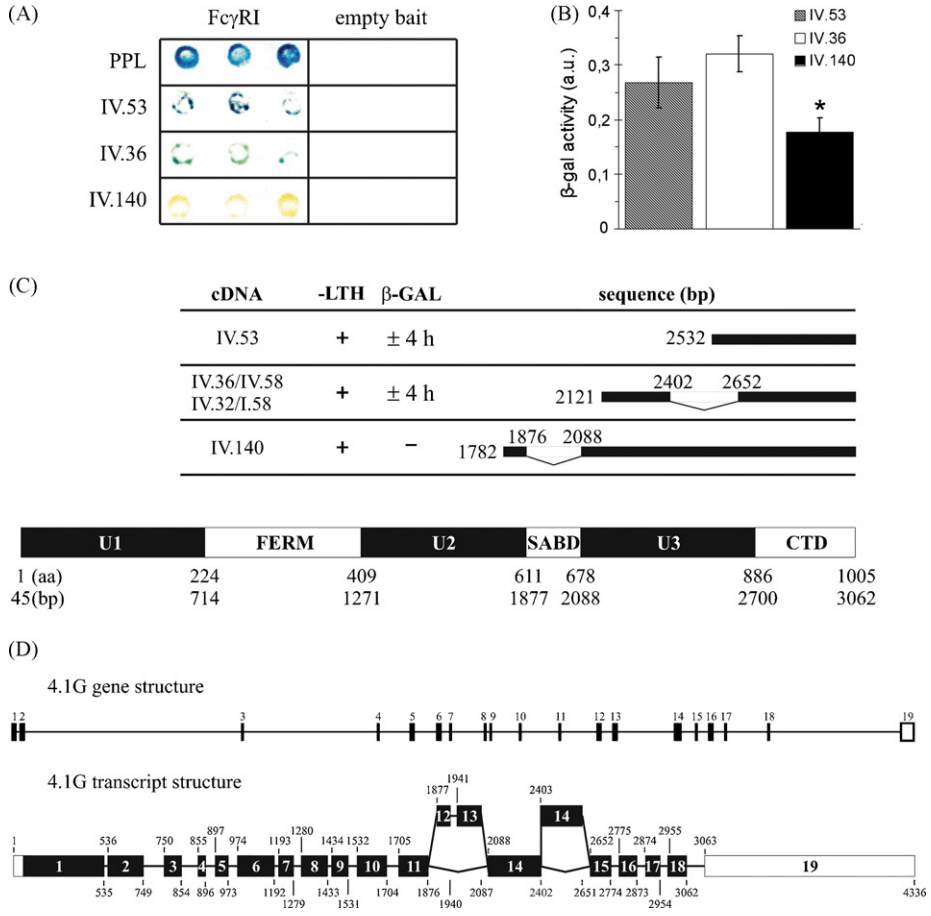

Fig. 1. Interaction of FcγRI with protein 4.1G splice variants. (A) Yeast colonies were assessed for growth on selective media (-LTH) and β-galactosidase activity upon transfection of FcγRI (or empty bait vector) and 4.1G cDNA in triplicate. Periplakin cDNA was used as positive control (Beekman et al., 2004a, $n = 3$). (B) Quantification of interaction strength for yeast clones IV.53, IV.36 and IV.140. Nine colonies per transfection were selected and tested for β-galactosidase activity in a liquid β-galactosidase assay using ONPG as substrate (protocol described in Clontech's Yeast Protocols Handbook). Data were averaged, error bars indicate standard deviation ($n = 2$). (C) Domain structure of full length 4.1G, and schematic presentation of six isolated cDNA. Growth on medium lacking LTH, and β-galactosidase activity (β-GAL, in hours before prominent blue staining was observed) are indicated. Spliced regions are marked by a white gap, and a cusped line. White boxes indicate conserved domains, whereas black boxes mark 4.1G unique domains (U1, U2, U3). Domain boundaries are indicated in amino acids or base pairs (counted according to GenBank accession number NM_001431). (D) Graphic view of 4.1G exon structure on genomic and transcript level. The different cDNA obtained by yeast two-hybrid assays were transposed on the exon structure of the 4.1G mRNA. Numbers above and below the sequence indicate the starting, and ending bp of associated exons, respectively. 4.1G spliced regions found in yeast two-hybrid sceens are indicated. Black boxes represent coding sequences and white boxes represent untranslated regions.

12 and 13 (SABD) resulting in a protein with 43 additional N-terminal residues compared to 4.1GΔ14 and a complete exon 14. The exon structure of the 4.1G mRNA and spliced fragments are schematically depicted in Fig. 1D (gene EPB41L2, GeneID: 2037, http://www.ncbi.nlm.nih.gov/entrez). These data suggested splice variants of 4.1G to selectively interact with FcγRI.

3.2. A 4.1G splice variant is predominantly expressed in immune cells

RT-PCR with 4.1G-specific primers that encompassed the 249 nt deletion fragment of exon 14 were used to discriminate between spliced 4.1G transcripts. Most immune cells tested showed expression of the 4.1GΔ14 splice-form, although DC, as well as MCF7 cells (human epithelial cell line), COS7 cells (monkey fibroblastic cell line) and IIA1.6 cells (mouse

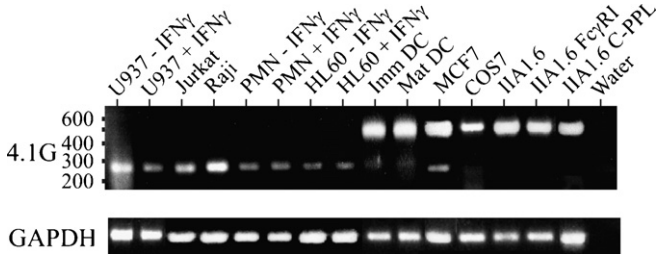

Fig. 2. Splicing of 4.1G transcripts in immune cells. 4.1G specific primers were designed that encompassed the 249 nt deleted region observed in 4.1G clones from yeast two-hybrid screens, and used to amplify cDNA derived from a panel of cells. IFN-γ stimulations of cells were performed overnight with 300 U/ml. Monocyte-derived immature dendritic cells (Imm DC, day 5) were cultured, and matured (Mat DC) as described in Section 2. FcγRI (IIA1.6 FcγRI) or C-terminal periplakin (IIA1.6 C-PPL)-transfected IIA1.6 cells were also tested. Products of 516 bp were expected for transcripts containing exon 14, whereas 267 bp products were expected in case of 4.1GΔ14-like transcripts. GAPDH was amplified as positive control.

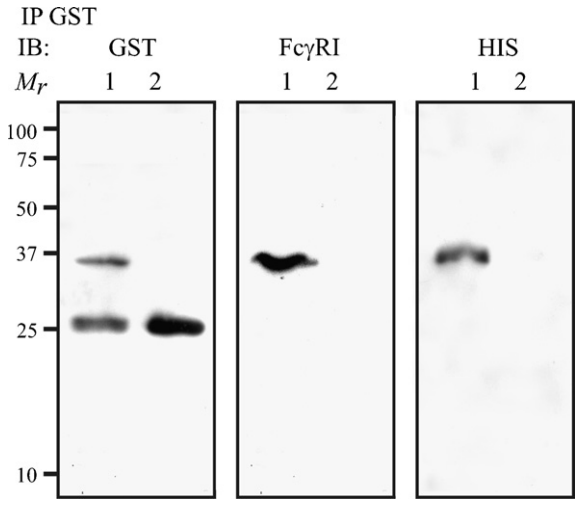

Fig. 3. Co-immunoprecipitation of recombinant GST-FcγRI and His-4.1G-CTDΔ14. Tosyl-activated beads were coated with goat polyclonal GST antibodies, and incubated overnight with GST-FcγRI (lane 1) or GST alone (lane 2) and His-4.1G-CTDΔ14 in RIPA buffer. Western blots of these fractions were stained with antibodies recognizing GST (left panel), FcγRI-CY (middle panel) or His-tag (right panel). One-out-of-three representative experiments is shown.

B/macrophage like cell line), were found positive for the full-length PCR fragment (Fig. 2). Incubation with IFN-γ (16 h), DC maturation or co-transfection with FcγRI or the C-terminus of periplakin – another FcγRI-CY associating protein (Beekman et al., 2004a) – did not alter the transcript profile. Sequence analyses of the two transcripts confirmed the 249 nt deletion that was also observed in the 4.1G cDNA identified in yeast two-hybrid screens (data not shown).

3.3. Recombinant FcγRI and 4.1G interact

To further assess the capacity of these proteins to interact, we expressed GST-tagged FcγRI-CY and His-tagged 4.1G clone I.58 (both with a predicted size of 35 kDa) in *E. coli* and performed immuno-precipitations with purified protein preparations. As indicated in the left panel of Fig. 3, anti-GST coated beads pulled down GST-FcγRI (lane 1) and GST (lane 2). The presence of FcγRI in lane 1 was confirmed by using an FcγRI-specific rabbit antiserum. Co-precipitation of His-4.1G was selectively observed with FcγRI-absorbed beads (right panel; Fig. 3). The His-tagged protein migrated slightly slower than FcγRI.

3.4. 4.1G binds to the membrane-proximal region of the FcγRI cytoplasmic tail

Cytosolic domains of activating FcR and truncated or alanine-substituted FcγRI mutants were used to better characterize the 4.1G interaction sites in yeast two-hybrid-binding assays. Growth of yeast colonies on selective media (-LTH) was per-

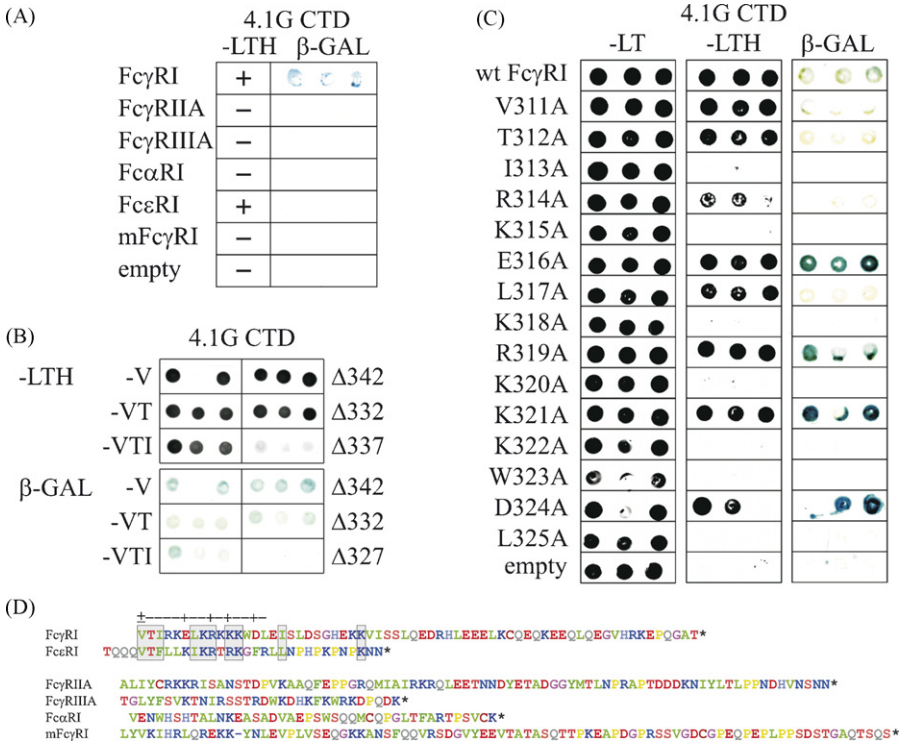

Fig. 4. Identification of protein 4.1G interaction sites in FcR cytoplasmic domains. Protein interactions were assessed after transfer of three yeast colonies to media without leucine, tryptophane (-LT) and histidine (-LTH) and a β-galactosidase (β-GAL) activity assay. (A) Interactions between a panel of activating FcR α-chain cytosolic domains, and 4.1GΔ14. mFcγRI denotes murine FcγRI. (B) Interactions between truncated FcγRI-CY mutants, and 4.1G-CTDΔ14. Numbers refer to the final C-terminal residue present in the FcγRI-truncated molecules. -V, -VT, -VTI constructs lack the first one, two or three N-terminal residues of FcγRI-CY. (C) Interaction of alanine-substituted FcγRI mutants with 4.1G-CTDΔ14. The single substitutions of FcγRI's membrane-proximal region (311–325) were denoted as V311A (valine at position 311 for alanine, T312A threonine for alanine at 312, etc). (D) Cytosolic domains of FcR. Similar regions within FcγRI and FcεRI are boxed in grey.

mitted after co-transformation of 4.1GΔ14 with FcγRI and FcεRI (Fig. 4A). Only human FcγRI induced sufficient levels of β-galactosidase. However, no interactions were observed for FcγRIIA, FcγRIIIA, FcαRI or mFcγRI. Various N and C-terminal-truncated mutants of FcγRI-CY were tested for interaction with 4.1G-CTDΔ14. Minimal binding requirements for 4.1G interaction were located within FcγRI-CY residues 312–332 as indicated by β-galactosidase assays (Fig. 4B). The importance of the FcγRI membrane-proximal region was further illustrated in experiments in which we replaced single residues of FcγRI-CY (311–325) for alanine (Fig. 4C). Most alanine substitutions abrogated interaction with 4.1G. Replacement of E316, R319, K321 and D324, however, resulted in increased β-galactosidase activity. Notably, FcγRI and FcεRI displayed considerable sequence similarity in their membrane-proximal domains (Fig. 4D).

3.5. 4.1G-interacting proteins share sequence similarities

To assess whether there are motifs within FcγRI that are shared with other 4.1G-CTD-binding proteins, we compared the primary sequence of FcγRI with other proteins that interact with 4.1G-CTD. These include transmembrane proteins such as platelet and T cell activation antigen 1 (PTA-1/CD226/DNAM-1) (Ralston et al., 2004), D2 and D3 dopamine receptors (D2DR, D3DR) (Binda et al., 2002), A1 adenosine receptor (A1AR) (Lu et al., 2004b), GluR-1 (Shen et al., 2000) and GluR-D AMPA receptors (Coleman et al., 2003). Nuclear mitotic apparatus protein (NuMa) (Delhommeau et al., 2002) and FK506-binding protein 13 (FKBP13) (Walensky et al., 1998) are cytosolic proteins that bind 4.1G. The membrane-proximal domains of these receptors showed significant sequence similarity and indicated a role for stretches of basic residues situated beneath the membrane (Fig. 5A). Cytosolic proteins FKBP13 and NuMa did not contain the large basic regions, but displayed somewhat similar patterns. The membrane-proximal region of FcγRI-CY is predicted to adopt an alpha helix, followed by loops and extended configurations (Fig. 5B). Similar structures were predicted for membrane-proximal regions of other 4.1G-binding proteins using PHD predictions (Rost, 1996) (Fig. 5C) and also for PTA-1 after submission to 3D-PSSM (Kelley et al., 2000) (result not shown). A schematic representation of FcγRI-residues 310–327 as alpha-helical wheel is displayed in Fig. 5D.

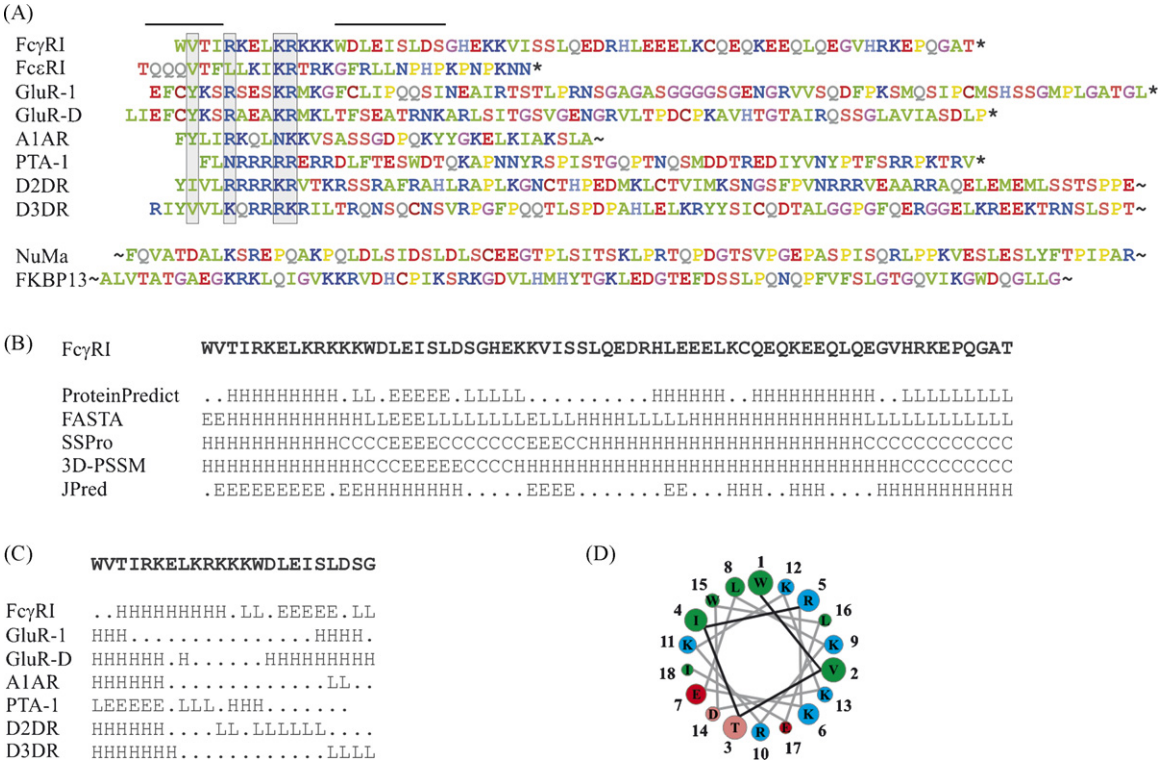

Fig. 5. Sequence analysis of 4.1G-interacting proteins. (A) Primary sequence alignment of 4.1G-CTD-binding proteins. Cytosolic domains of 4.1G-CTD-interacting proteins were predicted using PHDhtm prediction software (Rost, 1996), and similar regions were aligned. Transmembrane receptors are shown in the upper alignment (interacting loops are indicated for seven membrane-spanning receptors (dopamine receptors, A1AR)), and cytosolic proteins (not full sequences) in the lower alignment. Grey boxes mark similar residues within transmembrane receptors, allowing one mismatch. Regions of hydrophobic and (potentially) negatively charged residues are indicated by black lines. Amino acids in green, hydrophobic/aromatic; blue, basic/hydrophilic; red, acidic; yellow, aliphatic. (B) Secondary structures of FcγRI-CY were predicted after web submission of FcγRI (TM region and CY) to PredictProtein, FASTA, SSPro, 3D-PSSM and JPred. H indicates a predicted alpha-helix structure, E the extended β-sheet and C/L indicates coil/loop. Dots mark unreliable predictions. (C) Secondary structures of membrane-proximal domains of 4.1G-interacting protein. Regions were predicted with PredictProtein, and had cut-off accuracies of 82% (PHD prediction, Sub_sec). (D) Schematic presentation of an alpha-helical wheel for FcγRI residues 310–327.

4. Discussion

FcγRI cross-linking initiates signaling cascades that culminate in cellular functions. Although the cytosolic domains of most multi-subunit FcR are considered 'inert', interactions of FcγRI-CY with cytoskeletal-associated proteins such as periplakin and filamin A have been reported (Beekman et al., 2004a; Ohta et al., 1991). We now report the binding of FcγRI to protein 4.1G, a globular multi-domain protein that may act as signaling scaffold and tethers to the actin cytoskeleton. We found the FcγRI-CY membrane-proximal domain to interact with a conserved CTD within 4.1G in yeast two-hybrid screens.

The FcγRI 'docking-site' within 4.1G resided within residues 830–1005 (Fig. 1). The somewhat larger splice variant 4.1GΔ14 lacked residues 787–869, but interacted efficiently suggesting 4.1G residues 870–1005 to contain FcγRI-interacting capacity. Another spliced message (4.1GΔSABD) contained 43 additional N-terminal residues, and a complete exon 14 compared to 4.1GΔ14. This variant demonstrated a reduced capacity to interact with FcγRI-CY. This suggested the N-terminal 43 residues or the spliced region of exon 14 (or both) to carry 'inhibitory potential' for FcγRI-CY binding. Notably, the better FcγRI-interacting 4.1GΔ14 related transcripts were found enriched in most immune cells, suggesting alternative splicing of 4.1G to possibly regulate FcγRI interaction (Fig. 2). 4.1GΔ14 was, furthermore, confirmed to interact with FcγRI-CY by co-immunoprecipitation studies (Fig. 3). Tissue-specific splicing is commonly observed for 4.1 proteins and can be regulated by selective receptor–ligand interactions or phorbol esters, such as TPA (Conboy, 1999; Ralston et al., 2004). In Jurkat cells, TPA-induced upregulation of PTA-1 expression has been associated with selective expression of a 4.3 kb 4.1G splice-form (21). We, however, did not observe differences after stimulation with IFN-γ or upon DC maturation.

Our data indicated both FcγRI and FcεRI (albeit with reduced efficacy) to interact with 4.1G-CTDΔ14 in yeast cells and demonstrated a critical role for FcγRI membrane-proximal residues in this interaction (Fig. 4). Sequence alignments of 4.1G-binding domains of multiple proteins indicated stretches of hydrophobic residues (sometimes containing serines or threonines), followed by a basic cluster, and a less defined region of hydrophobic and (potentially) negative residues (S, T, D, E) to be important for interactions with 4.1G. Most of these proteins are predicted to enter the cytosol as alpha helices, but then to loop, and/or extend. Consensus residues (HxxBxxxBB, H represents hydrophobic residues, B basic residues (K/R), x any residue) within FcγRI and other 4.1G-CTD-interacting proteins were found in the predicted FcγRI alpha-helical structure. These aligments suggested a stretch of basic residues at one side of the helix (that have expected turns every 3.6 residues) to be a critical sequence pattern for 4.1G-CTD interaction. We, recently, also reported periplakin to interact with the FcγRI-CY membrane-proximal region, suggesting these proteins to possibly compete for FcγRI-CY (Beekman et al., 2004b).

Besides FcγRI and FcεRI, FcεRIIa and Fcα/μM bear membrane-proximal basic clusters, and to some extent FcγRI-similar adjacent sequences, and may interact with 4.1G for signaling (Shibuya et al., 2000; Yokota et al., 1988). Studies of dopamine receptors (Binda et al., 2002), A1AR (Lu et al., 2004b), AMPA receptors (Coleman et al., 2003; Shen et al., 2000), parathyroid hormone receptor (Saito et al., 2005) and metabotropic glutamate receptor mGlu1α (Lu et al., 2004a) indicated a prominent role for 4.1G interactions in stabilization of surface expression or signal transduction (PTA-1; Ralston et al., 2004, A1AR and mGlu1α; Lu et al., 2004a; Tateyama and Kubo, 2007). Other FERM domain-containing proteins have been shown implicated in formation of the immunological synapse by segregation of CD43 from the TCR area (Allenspach et al., 2001) and in B cell receptor induced lipid raft dynamics (Gupta et al., 2006). The impact of 4.1G interaction for FcγRI biology, however, remains to be determined.

Acknowledgements

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