Phage Display Based Cloning of Proteins Interacting with the Cytoplasmic Tail of Membrane Immunoglobulins*

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The reduced quantity and quality of serum immunoglobulins (sIgs) in mutant mice expressing truncated cytoplasmic tails of IgE and IgG1 indicate an active role for the cytoplasmic domains of mIgG1 and mIgE. We used phage display technology to identify candidate proteins able to interact with the cytoplasmic tail of IgE. Using a murine cDNA B cell library displayed on the surface of phage as prey and the 28 amino acid long cytoplasmic tail of IgE as bait, we isolated phage encoding the murine hematopoietic progenitor kinase 1 (HPK1). Surface plasmon resonance analysis measurements confirmed affinity of HPK1 to the mIgE cytoplasmic tail and revealed association to other immunoglobulin isotypes as well. Immunoprecipitation experiments, using lysates from two B cell lines expressing nitrophenyl (NP) specific mIgE molecules showed co-precipitation of IgE and HPK1. The interaction of HPK1 with the cytoplasmic domains of membrane immunoglobulins indicate an active role of the tails as part of an isotype specific signal transduction, independent from the Igα/Igβ heterodimers, and may represent a missing link to upstream regulatory elements of HPK1 activation.

Keywords: Antigen receptor; Cytoplasmic tail; HPK1; Phage display; Surface plasmon resonance

INTRODUCTION

The B cell antigen receptor (Vitetta et al., 1980; Reth, 1992) plays a central role in almost all processes along the developmental pathway of B cells, i.e. the generation, maturation, survival and activation of B cells. B cell antigen receptors (BCR) exist as a complex of membrane immunoglobulins (mIgs) and at least two other transmembrane polypeptides, called Ig-α and Ig-β, which connect the antigen receptor to the tyrosine phosphorylation pathways in the cell. In this complex, mIgs serve as isotype specific antigen receptors on the surface of B lymphocytes. All isotypes of mIgs can form a complex with Ig-α and Ig-β (Venkitaraman et al., 1991) indicating an involvement of all isotypes in the signal transduction pathway. The earliest detectable biochemical event that follows BCR-aggregation is increased activity of protein tyrosine kinases (PTKs) of the Syk- and Src-family, which phosphorylate distinct tyrosine residues within conserved sequences present on the cytoplasmic portions of Ig-α and Ig-β (Reth, 1992). Phosphorylation of Ig-α and Ig-β occurs on the tyrosine residues of immuno receptor tyrosine-based activation motif (ITAM) and on the non-ITAM tyrosine 204 of Ig-α (Engels et al., 2001). Once phosphorylated, ITAMs provide binding sites for SRC homology 2 (SH2) domains of the Src and Syk PTKs and the adaptor protein SLP-65 (Engels et al., 2001), thus initiating several signaling pathways, the most important of which are the PLC-γ, the Ras and the PI3-kinase pathways (Reth and Wienands, 1997; Kurosaki, 1999). Various external stimuli such as cytokine binding, interaction with other cells as well as stress factors including osmotic shock, heat or ionizing irradiation can influence the signaling cascade initiated by BCR engagement and they can contribute to differential alteration of the pattern of gene expression.

Obviously, between Ig subclasses, there should be no differences in the transduction of signals regulating the central features of the immune system (Nussenzweig, 1997) such as the control of allelic exclusion, early cellular transitions and in the function of the membrane-bound immunoglobulin as a receptor for antigen capture.
and presentation. However, it has been clearly shown (Achatz et al., 1997; Kaisho et al., 1997; Luger et al., 2001) that the cytoplasmic tails of IgG1 and IgE influence the quantity and quality of the immune response. These experiments show that, apart from the signal transduction pathways via Ig-α and Ig-β and an isotype-specific signal transduction pathway, initiated at the cytoplasmic tails of the mlgE, exists.

In the present work, we describe the use of the phage display technology for the isolation of candidate proteins able to interact with the cytoplasmic tail of mlgE and we present the murine hematopoietic progenitor kinase 1 (HPK1) (Kiefer et al., 1996), a 97 kDa serine/threonine kinase, as a candidate protein that could modify signaling events originating at the cytoplasmic tail of mlgE.

MATERIALS AND METHODS

Construction of the Phage Display B Cell Library

To allow cDNA cloning with EcoRI and XhoI as methylation sensitive restriction enzymes, pJuFo phagemid (Crameri and Suter, 1993) was re-modeled (Crameri et al., 2002) to pGA110. Splenocytes of 6 BALB/c mice were isolated and enriched for B cells (anti B220 monoclonal Ab RA3-6B2) yielding 3 x 10^7 cells. B cells were lysed and mRNA was isolated using the MACS mRNA kit. The 1st and 2nd cDNA strands were synthesized using the Stratagene cDNA synthesis kit according to the manufacturer’s instructions. For 1st strand cDNA synthesis 5 μg mRNA was primed with an oligo(dT) primer containing an internal XhoI restriction site (5’ GAGTCTG 3’). After 2nd strand synthesis, EcoRI adaptors were ligated to the blunted cDNA. cDNA inserts were ligated to pGA110 as EcoRI–XhoI fragments. Phages were generated as described before (Crameri and Suter, 1993; Crameri et al., 2002).

Coupling of the IgE Tail and Phage Panning

The IgE tail was synthesized as HIS-tagged peptide (HHHHHHHKVKWVFSTPMQDTPQTFQDYA-NILQTRA). One hundred microliters of 1 mg/ml peptide (solved in PBS with 10 μg/ml BSA) were added to Qiagen Ni-NTA (Hisorb-strips) wells. After incubation for 2 h at 37°C, the wells were washed twice with 100 μl PBS and residual sites were blocked with BSA (10 μg/ml). One hundred microliters of the phage library (10^11 phagemids) was added per coated Ni-NTA well and incubated for 2 h at 37°C. Five phage panning rounds were performed as described (Crameri et al., 2002).

Semiquantitative PCR Analysis

The relative amount of chPK-specific clones present after the 1st, 3rd and 5th panning round was determined by PCR using an chPK specific 3’-primer an a 5’-specific vector primer (cHPK1 3’-primer; 5’ GGAATTCATTGTCGTGATTGGAGTCTG 3’; pGA110 5’-primer; 5’ GCAAACGAAATCGCGAACCCTTG 3’). Equal numbers of phages (titer was determined before) after each panning round were used for re-infection of Escherichia coli, followed by subsequent plating on agar plates. Total colonies were washed off and phagemid DNA was extracted. PCR conditions were: 2 min 95°C primary denaturation; 20 cycles repeating 45 s 95°C, 45 s 59°C and 1 min 72°C; 10 min 72°C final extension.

Surface Plasmon Resonance Analysis

For surface plasmon resonance analysis two different sensor chips were used. (A): IgE-tail peptide (1 mg/ml diluted in PBS) was coupled to Flowchannel one (Fc1) of sensor chip CM5 (BIACORE) according to manufacturer’s instructions. 1683 response units (RUs) were coupled to Fc1. As reference, a comparable amount of a random peptide (1704 RUs) was coupled to channel two (Fc2). CM5 measurements were performed by injecting 10^5 cfu (suspended in TBS) for 1 min. Elution was done for 100 s. Phagemids were removed from the surface by injecting 100 mM NaOH.

(B): The NTA sensor chip was pre-treated and coupled as described by the manufacturer. One hundred microliters of HIS-tagged peptide (150 nM in 0.01 M Heps, 0.15 M NaCl, 50 μM EDTA and 0.005% surfactant P20) was injected. Measurements were performed by injecting phagemids (diluted to 10^5 cfu in TBS) for 1 min followed by 100 s of elution. The NTA sensor chip was regenerated for 5 min, followed by attachment of the next peptide. Four hundred (± 25) RUs were coupled for each HIS-tagged Ig-tail. Binding curves were calculated using the BIA-viewer software program.

Peptides:

IgG1: (6 x H) KVKWFSSVVELKQTLVPEYKNMIGQAP
IgG2a: (6 x H) KVKWFSSVVELKQTISPDYRNMIQGA
IgG2b: (6 x H) KVKWFSSVVELKQKISPDDYRNMIQGA
IgG3: (6 x H) KVKWFSSVQVKQTAIPDYNMIQGA
IgE: (6 x H) KVKWFSTPMQDTPQTFQDYA-NILQTRA
IgEΔTFQD: (6 x H) KVKWFSTPMQDTPQYA-NILQTRA
**B Cell Lysis, Immunoprecipitation and Co-immunoprecipitation**

JW813/4 cells were seeded at a concentration of $5 \times 10^4$ cells/ml culture. Aliquots for analysis were taken at days 5, 7, 10, 12, 14 and 17 of incubation. Plasmacytoma cells, $5 \times 10^7$ were harvested by centrifugation at 1200 rpm for 5 min and lysed on ice for 10 min in 1 ml of lysis buffer (50 mM Tris, 37 mM NaCl, 0.1–1% Digitonin, (pH = 7.6) containing 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM phenylmethysulfonyfluorid (PMSF) and 1 mM natriumorthovanadate). Insoluble material was removed by centrifugation at 10,000g for 10 min at 4°C. The supernatant, containing solubilized proteins, was stored at $-20^\circ$C until further analysis.

The B-cell lysate was divided into two aliquots of 0.5 ml each. One aliquot was incubated with 10 μl of NP-sepharose beads (Biosearch Technologies Inc.). The control aliquot was incubated with CL-2B sepharose beads. Both aliquots were incubated overnight at 4°C under constant gentle agitation. The immune complexes were centrifuged at 1000 rpm for 1 min. The supernatant was discarded and the sepharose beads were gently washed with 0.5 ml lysis buffer by inverting the tube five times. The last two steps were repeated twice. Samples were eluted from the beads by incubating with 50 μl of non-reducing 1 × SDS gel-loading buffer (6 mM Tris pH = 6.8 (with HCl), 2% SDS, 10% Glycerol, 0.1% Bromophenol blue) at room temperature for 30 min. The supernatant was loaded onto a SDS-polyacrylamide gel and transferred by electroblotting to an Immobilon-PVDF (polyvinylidine fluoride) membrane according to standard procedures. Narrow strips of the membrane were incubated with either AP-coupled rat anti mouse IgE (EM95-3), or rabbit anti HPK1 serum followed by incubation with a goat anti rabbit AP-coupled antibody (SIGMA).

**RESULTS**

**Isolation of Phagemids Displaying Proteins that Interact with the Cytoplasmic Tail of mIgE**

For the generation of phagemids, the phage display B cell library, constructed in pGA110 (Cramer et al., 2002), was superinfected with helper phage VCS M13. To guarantee full accessibility of the cytoplasmic tail during the phage display screening, the IgE tail was synthesized as HIS-tagged peptide and immobilized to Ni-NTA wells. Selective enrichment of phagemids was done as described before (Cramer and Suter, 1993). After five panning rounds phage enrichment was determined as the ratio between applied and eluted phage. More than 1000-fold enrichment was observed. Thirty randomly chosen single transductants from the last round of panning (fifth panning round) were sequenced. One specific sequence appeared several times from the last round of panning (fifth panning round) were observed. Thirty randomly chosen single transductants after the first, third and fifth panning round was determined by a semiquantitative PCR analysis using a cHPK specific 5′-primer and a 5′-specific vector primer. Selective enrichment of cHPK1 displaying phages, represented by a higher percentage of cHPK1 phagemids in the total phage population was observed, indicating specific interaction between cHPK1 protein and the cytoplasmic tail of IgE. (b) Specificity of the physical interaction between cHPK1-displaying phagemids and the cytoplasmic tail of IgE was investigated with surface plasmon resonance analysis, using a CM5 sensor chip with a covalently coupled IgE tail peptide. The whole library showed a weak, but measurable affinity for the IgE tail, indicating the existence of phages displaying proteins with specificity for the IgE tail. In contrast, the cHPK1 displayed protein showed a remarkable increase of affinity for the cytoplasmic tail of IgE. cHPK1, 1 × 10^7, displaying phages were injected over a period of 200 s. After the elution step, a relative increase in affinity of 500 RUs could be measured.

**FIGURE 1** (a) The relative amount of cHPK-specific amplification product present after the 1st, 3rd and 5th panning round was determined by a semiquantitative PCR analysis using a cHPK specific 5′-primer and a 5′-specific vector primer. Selective enrichment of cHPK1 displaying phages, represented by a higher percentage of cHPK1 phagemids in the total phage population was observed, indicating specific interaction between cHPK1 protein and the cytoplasmic tail of IgE. The complete phage display cDNA library served as control. The whole library showed a weak, but measurable affinity for the IgE tail, indicating the existence of phages displaying proteins with specificity for the IgE tail. In contrast, the cHPK1 displayed protein showed a remarkable increase of affinity for the cytoplasmic tail of IgE. cHPK1, 1 × 10^7, displaying phages were injected over a period of 200 s. After the elution step, a relative increase in affinity of 500 RUs could be measured.
cHPK1 and the total B cell cDNA library were harvested, and titers were adjusted to $1 \times 10^{10}$ cfu/ml each. One hundred microliters of phagemid suspension ($1 \times 10^9$ cfu) was injected over a period of 200 s, followed by subsequent washing with TBS. The binding curves indicated a high relative affinity of cHPK1-carrying phagemids and a measurable affinity for the whole B-cell library.

**Binding of cHPK1 to Cytoplasmic Tails of other Isotypes**

We next determined whether cHPK1 and cytoplasmic Ig-tails other than IgE could interact. HIS-tagged cytoplasmic tails of the IgM, IgG3, IgG1, IgG2a, IgG2b and IgE were synthesized and subsequently coupled to a Ni$_2^+$-NTA sensor chip via the HIS-tag. In comparison to the wild type IgE tail, the binding capacity of IgE$\Delta$TFQD is decreased by 47%, indicating a dramatic effect of the amino acid deletion on the binding affinity (Table I).

![Amino acid sequence alignment of the cytoplasmic tails of IgM, IgG3, IgG1, IgG2a, IgG2b and IgE. Amino acid residues that match IgE exactly are boxed.](image)

![Binding curves for the binding of cHPK1 to IgE and IgE$\Delta$TFQD.](image)

### Table 1

| Ig-Isotype     | Injection after 60 s | Elution after 100 s | %   |
|----------------|----------------------|---------------------|-----|
| IgE            | 691                  | 177                 | 100 |
| IgE$\Delta$TFQD | 563                  | 94                  | 53  |
| IgG1           | 819                  | 128                 | 72  |
| IgG2a          | 284                  | 68                  | 38  |
| IgG2b          | 276                  | 72                  | 41  |
| IgG3           | 744                  | 120                 | 67  |
| IgM            | 617                  | $\equiv0$           | $\equiv0$ |
| Random peptide | 310                  | $\equiv0$           | $\equiv0$ |

The same amount of peptide (RU) was covalently coupled to a CM5 sensor chip. Binding values (response units, RU) were taken 60 s after injection and 100 s after elution. IgE values were set to 100%.
HPK1 Co-precipitates with mlgE Expressing Plasmacytomas

The lymphoid myeloma lines J558L-Em and JW813/4 express mlgE molecules with specificity for the hapten 4-hydroxy-3-nitrophenylacetyl (NP). Both cell lines were stably transfected with a mlgE expressing genomic construct. However, after fresh splitting of the cell culture IgE surface expression can only be observed during a small time window. To find the best conditions for mlgE- and HPK1 expression in the JW813/4 cell line, cells were monitored over a period of 17 days using FACS analysis with FITC-coupled α-IgE (EM95-3) (Fig. 3a) antibodies and immunoblotting for detection of IgE and HPK1 (Fig. 3b).

For FACS analysis, 10^6 cells were labeled with FITC-coupled α-IgE (EM95/3) antibodies and the number of cells expressing mlgE was measured (Fig. 3a). mlgE surface expression peaked at day 10. For immunoblotting aliquots of 2 x 10^6 cells were collected for each time point and lysed in 200 µl of lysis buffer. Ten microliters of each sample was separated by SDS-PAGE under non-denaturing conditions, transferred to polyvinyl membranes and probed for IgE and HPK1 presence (Fig. 3b). While HPK1 expression decreased during the time course, IgE expression reached a maximum at day 12, followed by a rapid decrease. Because membrane bound IgE is evidently the most crucial parameter for co-precipitation, we decided to continue experiments with cells from 10-day-old cultures. This time point is a compromise between surface expression of mlgE, and an acceptable level of HPK1 expression.

Immunoprecipitation and co-immunoprecipitation were performed with J558L-Em and JW813 cell lysates. We failed to co-precipitate mlgE with HPK1 when digitonin in a concentration of 1 or 0.5% was used. Best results were observed with a 0.1% digitonin concentration. In Fig. 3c, results of a co-precipitation experiment with JW813 cells under these conditions are depicted. Lysates of JW813 cells (5 x 10^7 cells in 1 ml lysis buffer) were incubated with NP-Sepharose or control Sepharose. After precipitation complexes were separated on non-reducing 4-12% gradient SDS-PAGE. The samples were blotted on a nitrocellulose membrane and probed for IgE and HPK1 presence (Fig. 3c). The results were clearly visible for IgE and HPK1. HPK1 precipitated with IgE, proving a physical interaction between the two molecules.

DISCUSSION

In the present work, we described the use of the phage display technology to isolate candidate proteins able to interact with the cytoplasmic tail of mlgE. Several theoretical objections could be raised against using this technology for finding interacting proteins. First, the phF6o system is a prokaryotic system. Proteins that are presented on the surface of the phage may not be folded properly and therefore have a decreased specificity due to missing modifications. Second, the putative ligand could need phosphorylated residues in order to bind,
FIG. 3 (a) mIgE surface expression in the JW813/4 cell line was monitored over a period of 17 days. Flow cytometric analysis was performed with FITC-coupled α-IgE EM95-3 antibodies. Dead cells were excluded using propidium iodide. mIgE expression peaked at day 10. (b) Immunoblotting experiment for the detection of IgE and HPK1 protein in the cell line. IgE expression nearly matched with the FACS experiment (a) and peaked at day 12, while HPK1 protein expression showed the maximum level at day 5. For the co-immunoprecipitation 10-days-old cells were used. (c) JW813 cells were lysed and precipitated with NP-sepharose and control sepharose. Both immune complexes were separated on a non-reducing polyacrylamide gel and transferred onto a PVDF-membrane and developed with AP-coupled rat anti mouse IgE (EM95-3), or rabbit anti HPK1 serum followed by incubation with a goat anti rabbit AP-coupled antibody. Both HPK1 and mIgE were detected in the same precipitate.
which was not provided by the synthetic IgE tail peptide. Third, the vicinity of the Igα/Igβ sheath could be crucial for the interaction. Finally, a potential binding partner might need the vicinity of the “dimeric” tail, as it naturally occurs in the cell, for efficient interaction. We point to this issue, because we failed to isolate candidate genes using the yeast-two-hybrid system. We reasoned that the phage display system could resolve this problem: HIS-tagged peptides, bound to NTA (nitrilo-triacetic-acid)-wells, theoretically can float freely in solution and thus present dimeric motifs to possible interacting partners during the selection procedure.

Indeed, we were able to isolate a potential candidate, the HPK1. The specific interaction of HPK1 with the cytoplasmic tail of IgE was demonstrated in different and independent experiments. Using surface plasmon resonance analysis, we could show that cHPK1 directly interacts with the cytoplasmic tail of IgE. However, with the exception of IgM, HPK1 interacted also with the cytoplasmic domains of other Ig isotypes, albeit with a lower affinity. This observation raises the possibility that isotype-dependent signal transduction pathways exist in parallel to the well-characterized signal transduction pathways via the Igα/Igβ sheath proteins.

The insert isolated from the phages encoded 87 amino acids that fully matched the carboxy-terminal part of the murine (HPK1) (Kiefer et al., 1996), a 97-kDa serine/threonine kinase. A serine/threonine kinase binding motif (S/T-x-x-D/E) earlier identified by Litchfield et al. (1990) is present in the IgE tail (T-F-Q-D) and could serve as a docking site for HPK1. A similar motif is also present in IgG1, IgG2b and IgG2a as amino acid sequence S-V-V-Q. Only IgG3 lacks this consensus or uses a modified version in form of S-V-V-Q. To show the importance of this binding site for the interaction with HPK1 we used a modified IgE tail, lacking the four amino acids and repeated the BIACORE affinity measurements. The affinity of HPK1 for the modified IgE tail was dramatically reduced; a 47% reduction of affinity was measured, indicating the importance of the T-F-Q-D motif in the binding of HPK1 to the tail of IgE.

Our results indicate that the interaction of HPK1 and the cytoplasmic tail of IgE are phosphorylation independent, and additionally independent of the vicinity of the Igα/Igβ sheath. We point to this issue because the recently identified B cell specific adaptor protein SLP 65 (BASH or BLNK) (Fu et al., 1998; Goitiska et al., 1998; Wiemands et al., 1998) containing SH2 domains, was shown to interact physically with the HPK1 protein in a phosphorylation-dependent manner (Sauer et al., 1997; Tsui et al., 2001). However, this interaction was only visible after stimulation of the surface antigen receptor with anti BCR specific antibodies. To mimic a situation which resembles the original cloning experiment and the BIACORE-measurements, our experiments were performed with unstimulated mIgE expressing cell lines.

HPK1 was shown to play a central role in the network of cytoplasmic signaling in mice. HPK1 is expressed in nearly all embryonic tissues, but becomes restricted to hematopoietic organs (bone marrow, spleen, thymus) in adult mice. HPK1 belongs to the family of germininal center kinases (GCK), which all feature a proline-rich C-terminal regulatory domain (Liou et al., 2000) and a N-terminal kinase domain, which shows homology to the yeast pheromone pathway element STE20. Although the physiological relevance of HPK1 remains to be determined, several factors promoting HPK1 activation have already been identified. These activators include interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β) (Zhou et al., 1999), erythropoietin (Nagata et al., 1999), epidermal growth factor (EGF) (Anafi et al., 1997), cellular injury (heat, UV and ionizing irradiation, chemotherapeutic drugs) and osmotic shock (Kiefer et al., 1996). Furthermore, in vitro assays revealed potent autophosphorylation of HPK1 (Kiefer et al., 1996).

Upstream elements, which finally lead to HPK1 activation are still poorly understood. HPK1 is suggested to act immediately downstream of membrane proximal signaling elements such as transmembrane receptors or small G-proteins (Kiefer et al., 1996). Binding to tyrosine kinases is suggested as well (Anafi et al., 1997). Most likely, translocation to membrane elements is accomplished by adaptor proteins containing src-homology 2 and 3 domains (SH2, SH3), which exhibit the potential to interact with the proline rich motifs of the C-terminal portion of HPK1 (Anafi et al., 1997; Oehrli et al., 1998; Ensenat et al., 1999). The C-terminal binding motifs also play a crucial role in the connection of HPK1 with downstream signaling elements, which are definitely better investigated than their upstream counterparts. Based on their homology to the yeast pheromone pathway kinase STE20, the GCKs may link receptor activation to the regulation of mitogen activated protein kinase (MAPK) modules. Indeed, HPK1 could be identified as potent upstream activator of the c-jun N-terminal kinase (JNK) pathway via the phosphorylation of the MAPK kinase kinase (MAPKKK) MLK-3 (Kiefer et al., 1996).

Because caspases regulate the JNK pathway (Chen et al., 1999), HPK1 activation could also depend on caspase-mediated cleavage. Beyond c-jun activation, HPK1 was shown to be involved in another central immune specific signaling pathway, which leads to activation of nuclear factor kappa-B (NF-κB) (Hu et al., 1999). While some of the MAPKKKKs preferentially act on a single MAPK module, others may stimulate more than one pathway. Thus, the existence of a broad spectrum of possible substrates for HPK1 may not be excluded.

In the present paper, we indicate the existence of a signaling cascade facilitated by the binding of unphosphorylated HPK1 to the cytoplasmic tails of membrane-bound immunoglobulins. Possibly, this additionally modifies the signal in either quality or quantity, thereby modifying downstream signaling events and gene
expression. We further propose that the physical interaction between the cytoplasmic tails of mlgS and HPK1 represents the missing link to upstream regulatory elements of HPK1.

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