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# RACK1 Protein Interacts with *Helicobacter pylori* VacA Cytotoxin: The Yeast Two-Hybrid Approach

Ewa E. Hennig,[1] Eugeniusz Butruk, and Jerzy Ostrowski
*Department of Gastroenterology, Medical Center for Postgraduate Education, Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology, 02-781 Warsaw, Poland*

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**The VacA toxin is the major virulence factor of *Helicobacter pylori*. The studies on VacA intracellular expression suggest that it interacts with cytosolic proteins and that this interaction contributes significantly to vacuolization. The aim of this study was to identify the host protein(s) that interacts with the VacA protein. We used the fragments of VacA protein fused with GAL4-BD as the baits in the yeast two-hybrid approach. The yeast transformed with plasmids encoding bait proteins were screened with human gastric mucosa cDNA library, encoded C-terminal fusion proteins with GAL4-AD. Three independent His-β-Gal-positive clones were identified in VacA-b1 screen; they matched two different lengths of cDNA encoding RACK1 protein. The specific activity of β-galactosidase found in the yeast expressing both VacA-b1 and RACK1 fusion proteins was 12–19 times higher compared to all negative controls used. VacA is capable of binding the RACK1 *in vitro* as was confirmed by the pull-down assay with GST fusion VacA protein and [$^{35}$S]Met-labeled RACK1 protein fragments.** 

***Key Words: Helicobacter pylori*; VacA cytotoxin; RACK1; two-hybrid system.**

*Helicobacter pylori* (*H. pylori*), the microorganism that colonizes human gastric mucosa of more than half of the world's population, has been identified as an etiological factor of such different disorders as chronic gastritis, duodenal and gastric peptic ulcer, gastric adenocarcinoma, and MALT lymphoma (1–4). Although the mechanisms responsible for the diversity of clinical outcomes of *H. pylori* infection are still not well established, both bacterial and host genetic factors and their specific interactions are suspected to play a role in the development of *H. pylori*-related disorders.

The vacuolating cytotoxin VacA is thought to be the major virulence factor of *H. pylori*. It is synthesized as a 140-kDa precursor and following proteolytic processing is secreted by bacterial cells (5–7). The mature 88-kDa toxin, comprising N-terminal (p33) and C-terminal (p55) domains, forms oligomeric structures with either six- or sevenfold radial symmetry (8, 9).

Secreted VacA interacts with the surface of target cells by binding to multiple cell surface proteins such as a 250-kDa receptor protein tyrosine phosphatase (RPTPβ on AZ-521 cells (10), an unidentified 140-kDa protein of a variety of epithelial cell lines (11), and a 170-kDa epidermal growth factor (EGF) receptor on HeLa cells (12). However, there is not a single high-affinity receptor for VacA. Toxin internalization and translocation into the cytosol results in the formation of intracellular vacuoles originating from late endosomal and lysosomal compartments of eukaryotic cells (13–15). This vacuolating activity of VacA depends on disassembly of the toxin oligomers upon acidic or alkaline pH conditions (10, 16, 17), that allows for toxin efficient internalization (18), as well as formation of anion-selective channels in lipid bilayers and cell plasma membrane (19–21).

The studies on VacA intracellular expression (22–24) suggest that the vacuole formation is likely a consequence of toxin interaction with cytosolic protein(s) of target cells. Vacuolization process requires an activity of a vacuolar-type ATPase, rab7 and Rac1 GTP-ase (14, 25, 26). Recently, the novel intermediate filament protein VIP54 that interacts with vimentin, was identified as VacA-interacting protein of 54 kDa in HeLa cells (27). However, the molecular mechanism of VacA-induced vacuole formation still remains unknown.

Proteins are usually the functional molecules and protein–protein interactions are critical to most biological processes. Among different methods that facilitate the analysis of the proteins interactions, the yeast two-hybrid system utilizes a molecular genetic approach

[1] To whom the correspondence and reprint requests should be addressed at Department of Gastroenterology, Medical Center for Postgraduate Education in Cancer Center, Roentgena 5 str, 02-781 Warsaw, Poland. Fax: (48-22) 644-7601. E-mail: hennige@coi.waw.pl.



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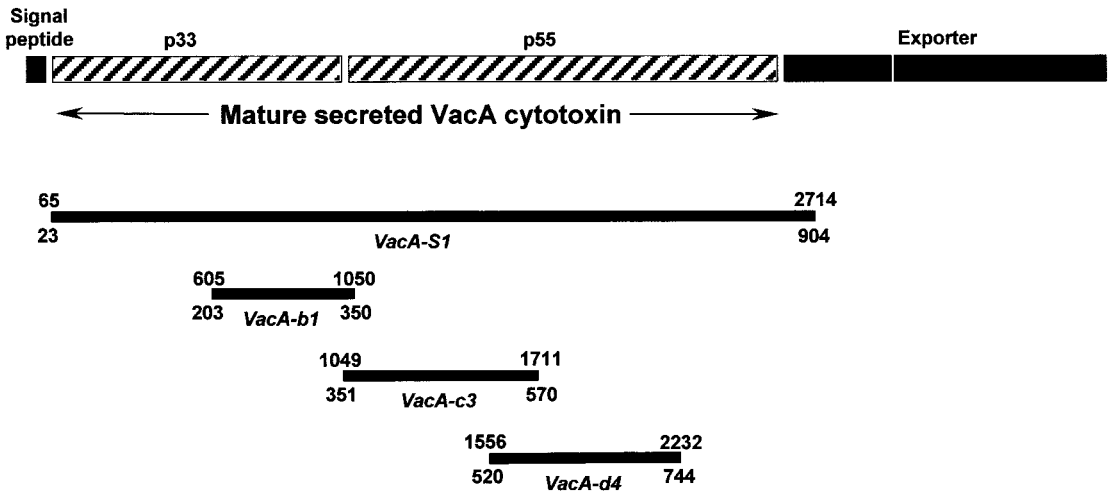


**FIG. 1.** The *vacA* gene fragments amplified by PCR and used throughout the study. Numbers of N-terminal and C-terminal nucleotides (above) and amino acids (below) of each fragment are indicated.

detecting protein–protein interactions in the native conditions of the cell and allows for selection of genes encoding potential interacting proteins (28).

The aim of this study was to identify the host protein(s) of gastric mucosa, which interacts with the VacA protein. For this purpose, we have constructed the two-hybrid cDNA library that encoded human gastric proteins and screen it with different fragments of VacA protein as the baits. We have identified RACK1, a receptor for activated C kinase (PKC) and a homolog of the heterotrimeric G-protein $\beta$ subunit, as VacA-binding protein.

## MATERIALS AND METHODS

*Materials.* HybriZAP-2.1 two-hybrid cDNA synthesis and Gigapack cloning kits (Stratagene, La Jolla, CA) were used in the study.

*Construction of human gastric mucosa cDNA library.* To construct cDNA library, total RNA was isolated from normal gastric mucosa, which was taken from surgically resected stomach of patient with gastric adenocarcinoma, using TRIzol Reagent according to manufacturer's protocol (Gibco BRL, Grand Island, NY). Then, poly($A^+$) RNA was separated by twice pass through oligo(dT) cellulose column. cDNAs were synthesized using the hybrid oligo(dT) linker-primer that contains an *Xho*I restriction site and cDNA Synthesis System. The double-stranded cDNA termini were blunted with *Pfu* DNA polymerase and *Eco*RI adapters were ligated. Following *Xho*I digestion, the cDNA fragments were size fractionated by electrophoresis on agarose gel. The 400- to 800-bp fragments were purified from the gel on the SpindBind columns (FMC BioProducts, Rockland, ME) and ligated into HybriZAP-2.1 vector, predigested with *Eco*RI and *Xho*I. $10^{10}$ cfu/ml of pAD-GAL4 phagemid library that encoded C-terminal fusion proteins with GAL4 activation domain (AD) were obtained by the mass *in vivo* excision of the entire HybriZAP-2.1 library, according to manufacturer's protocol.

*Construction of pBD-VacA yeast expression plasmids.* The relevant fragments of *vacA* gene from genomic DNA of *H. pylori* strain (typed as s1a/m1, according to Atherton *et al.*, 29) isolated from patient with duodenal ulcer disease were amplified by PCR using following oligonucleotides: 5′-TTCCCGGGAAATCAATAATCGTGTGG and 5′-CTCTTGTTTGTCGTTTTTAGCA for *vacA*-b1; 5′-CACCCGGGAGAGCAGTCAAAACAATA and 5′-TGCCTATATCTTCGCTAAAATG for *vacA*-c3; 5′-CACCCGGGTAGATTTTAGTGGCGTTA and 5′-GTCAGTGCCCGCAGAAACAT for *vacA*-d4; 5′-AGCCCGGGCATTAGTCAGCATCACAC and 5′-CTGCCTTGAGCGCCTGAGTTAG for *vacA*-S1, with the recognition sequence (underlined) for *Sma*I. The resulting PCR products (Fig. 1) were ligated into pCR2.1-TOPO vector (Invitrogen Corp., San Diego, CA). Following digestion with *Sma*I and *Xho*I and gel purification, inserts were subcloned into pBD-GAL4 Cam phagemid vector (Stratagene, La Jolla, CA) previously digested with *Sma*I and *Sal*I. All plasmid constructs were verified by nucleotide sequencing. Full sequences of *vacA*-b1 and *vacA*-c3 fragments, which were used for two-hybrid screens, are presented in Fig. 2 in comparison to *vacA* gene relevant sequences of *H. pylori* 60190 strain (ATCC 49503). The expression of encoded hybrid VacA bait proteins fused with GAL4 DNA-binding domain (BD) was determined by Western blot analysis with mouse monoclonal GAL4 (DBD) antibody (Santa Cruz Biotech, Santa Cruz, CA) in Epicurian Coli XL1-Blue (Stratagene, La Jolla, CA) bacterial host (data not shown).

*Yeast two-hybrid screens.* *Saccharomyces cerevisiae* strain YRG-2 (Stratagene, La Jolla, CA) was transformed with pBD-VacA-b1 or pBD-VacA-c3 plasmids by the method of Chen *et al.* (30). The screening procedure for isolation of human proteins interacting with VacA protein fragments was essentially performed according to the manufacturer's protocol (Stratagene, La Jolla, CA). Briefly, the yeast strain expressed VacA-b1 or VacA-c3 bait protein was transformed with human gastric mucosa cDNA library by the lithium acetate method. To screen the cDNA library, positive clones were initially selected for histidine prototrophy and then assayed for *lacZ* activity using a filter $\beta$-galactosidase assay with 5-bromo-4-chloro-3-indolyl-$\beta$-D-galactopyranoside (X-Gal). Plasmids were isolated from positive yeast clones and transformed into *Escherichia coli* DH5$\alpha$ for further analysis of insert size and sequencing.

*Quantitative assay of $\beta$-galactosidase activity.* Yeast strains were grown and assayed according to manufacturer's protocol (Yeast Protocols Handbook; Clontech Laboratories, Inc., Palo Alto, CA). Briefly, yeast cultures were grown to stationary phase in selection medium lacking leucine and tryptophan, diluted 1:5 in YPD medium (Sigma Chemical Co., St. Louis, MO) and incubated again until mid-log phase. The $\beta$-gal activity was determined in liquid yeast cultures using colorimetric assay with *o*-nitrophenyl-$\beta$-D-galactopyranoside (ONPG). The protein concentration of the cells was determined using Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA).

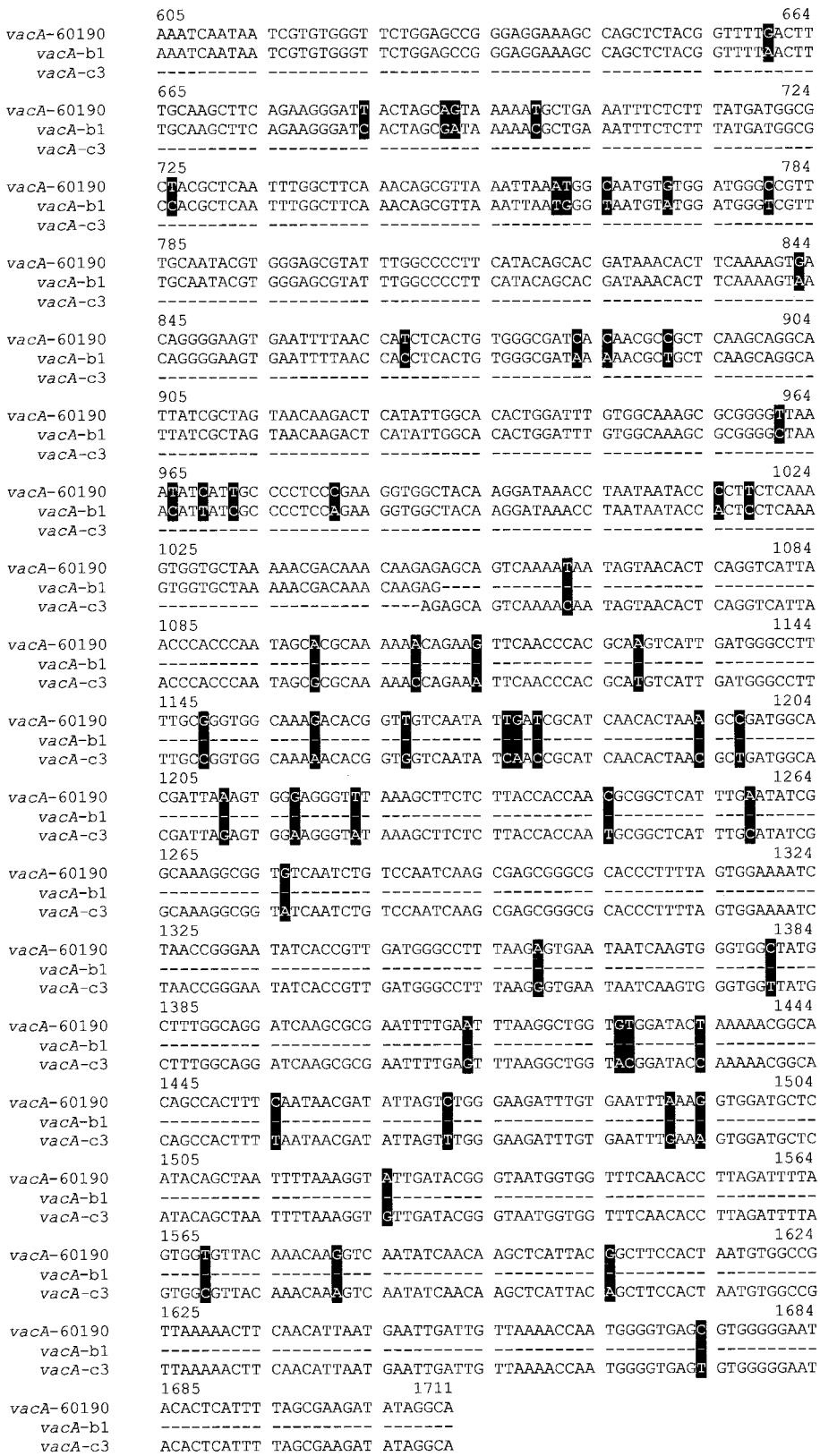


**FIG. 2.** Comparison of sequences of *vacA*-b1 and *vacA*-c3 fragments, used in this study, with relevant *vacA* sequence of *H. pylori* 60190 strain (GenBank Accession No. U05676). The numbers indicate the nucleotide positions in coding region of the gene. Differing bases have been marked.

*Expression of glutathione-S-transferase (GST) fusion proteins.* For *in vitro* binding experiments, *vacA*-b1 and *vacA*-S1 fragments were subcloned into pGEX-4T-3 vector and expressed in *E. coli* BL21 (Amersham Pharmacia Biotech, Vienna, Austria) as GST fusions. Bacteria were harvested, washed with phosphate-buffered saline (PBS), resuspended in lysis buffer (1% *N*-lauroyl-sarcosine in PBS with protease inhibitors), and sonicated on ice. After centrifugation to remove cell debris, 2% Triton X-100 was added. Fusion proteins were purified from lysates by adsorption to glutathione–Sepharose 4B beads (Amersham Pharmacia Biotech, Vienna, Austria) and used as a matrix for *in vitro* binding after several washings with PBS containing protease inhibitors. Expression of GST fusion proteins was confirmed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and Coomassie blue staining.

*In vitro translation and GST fusion protein binding.* cDNAs encoded fragments of RACK1 protein, from two-hybrid positive clones pAD-RACK1-31/1 and pAD-RACK1-8/1, were subcloned into pGEM-3Zf(+) vector (Promega Corp., Madison, WI) under control of SP6 RNA polymerase promoter and expressed in *E. coli* DH5$\alpha$. [$^{35}$S]Met-labeled RACK1-31/1 and RACK1-8/1 proteins were generated by using TnT Coupled Reticulocyte Lysate System (Promega Corp., Madison, WI) as instructed by manufacturer. One-fifth of *in vitro* translation products was incubated in PBS for 2 h at RT with GST fusion VacA protein fragments bound to glutathione–Sepharose matrix. After vigorous washing with NETN buffer (100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 20 mM Tris, pH 8.0), bound proteins were separated by SDS–PAGE and visualized by autoradiography.

*DNA sequencing analysis.* DNA sequences were determined using ABI PRISM BigDye terminator cycle sequencing ready reaction kit according to manufacturer's instructions (PE Biosystems, Foster City, CA). Nucleotide sequence databases were searched for homologous sequences with BLAST search analysis (www.ncbi.nlm.nih.gov).

*Statistical analysis.* All data are reported as mean ± SD. The results were compared statistically by using Student's test for unpaired data. Significance was indicated by a two-tailed *P*-value of less than 0.05.

## RESULTS

### *Isolation of RACK1 as a Protein Interacting with VacA-b1 in a Two-Hybrid Screen*

The yeast two-hybrid approach was used to identify human proteins that interact with the *H. pylori* VacA protein. For this purpose, we constructed a phagemid library of human gastric mucosa cDNA encoding C-terminal fusion proteins with the transcriptional AD of GAL4, and screened this library with fragments of VacA protein fused with GAL4-BD as a bait. First, the plasmid encoding bait protein was introduced into two-hybrid YRG2 yeast strain that contains both *HIS3* and *lacZ* reporter genes under the regulation of GAL4. This screening strain was subsequently transformed with human gastric mucosa cDNA library. Transformants were selected on histidine deficient synthetic medium for clones expressed *HIS3* reporter gene. Those clones were then tested by $\beta$-galactosidase assay for *lacZ* reporter gene expression.

The VacA-b1 (C-terminal region of p33) and VacA-c3 (N-terminal region of p55) fragments were used for the screenings (Fig. 1), and $1.6 \times 10^6$ transformants were analyzed with each bait. None positive clones were identified for VacA-c3 bait. The results of VacA-b1 screen are summarized in Table 1. Plasmids from 41 independent His-$\beta$-Gal-positive yeast colonies, isolated and transformed into *E. coli*, were passed through secondary screen in order to eliminate false positive interactions. Finally, 3 independent clones were identified. Sequence analysis followed by database search revealed that they matched two different length of cDNA encoding C-terminal fragment of the same protein; receptor for activated C-kinase (RACK1), also described as a homolog of the $\beta$ subunit of G proteins (31). RACK1 is a 36-kDa protein consisting of 317 amino acids (aa) that form seven repeating units of Trp-Asp (WD) motifs (32). The cDNA inserts from positive clones coded peptides from 178–317 aa (clone 31/1) and 197–317 aa (both clones 7/5 and 8/1) of RACK1 protein (Fig. 3A).

**TABLE 1**

The Results of a Two-Hybrid Screen with VacA-b1 Fragment as a Bait Protein

| | |
|---|---|
| Number of transformants screened | 1,600,000 |
| Number of His$^+$ and LacZ$^+$ colonies in primary screen | 41 |
| Number of plasmids sequenced | 27 |
| Number of His$^+$ and LacZ$^+$ clones in secondary screen | 3 |
| Number of different mRNAs | 1 |

### *Specificity of the RACK1 and VacA-b1 Interaction in Yeast Cells*

In order to examine the specificity of interactions between VacA-b1 and RACK1 protein fragments, the pairs of different pAD and pBD plasmids, including manufacturer's (Stratagene) positive control (PC) (plasmids p53-BD and pSV40-AD) and negative control (NC) (plasmids pLaminC-BD and pSV40-AD), were co-transformed into YRG2 strain. The pAD plasmids contained the following inserts: RACK1-31/1 or RACK1-8/1 or none, and pBD plasmids contained inserts: VacA-b1 or VacA-d4 or none. Transformed strains were plated on the medium lacking histidine (His$^-$) and supplemented with this amino acid (His$^+$). Only clones carried plasmids encoding VacA-b1 and RACK1-31/1 or RACK1-8/1 as well as PC were able to growth on both types of plates (an example shown in Fig. 3B).

The described above yeast strains were also used to analyze the stringency of identified interactions. The quantitative assay of $\beta$-galactosidase activity was performed in liquid yeast cultures. As is shown in Fig. 3C, the specific activities of $\beta$-gal measured in cells expressing both bait VacA-b1 and RACK1 protein fragments were 12–19 times higher comparing to all used negative controls ($P < 0.0001$). The level of enzyme activity was comparable with that obtain in clones of PC.

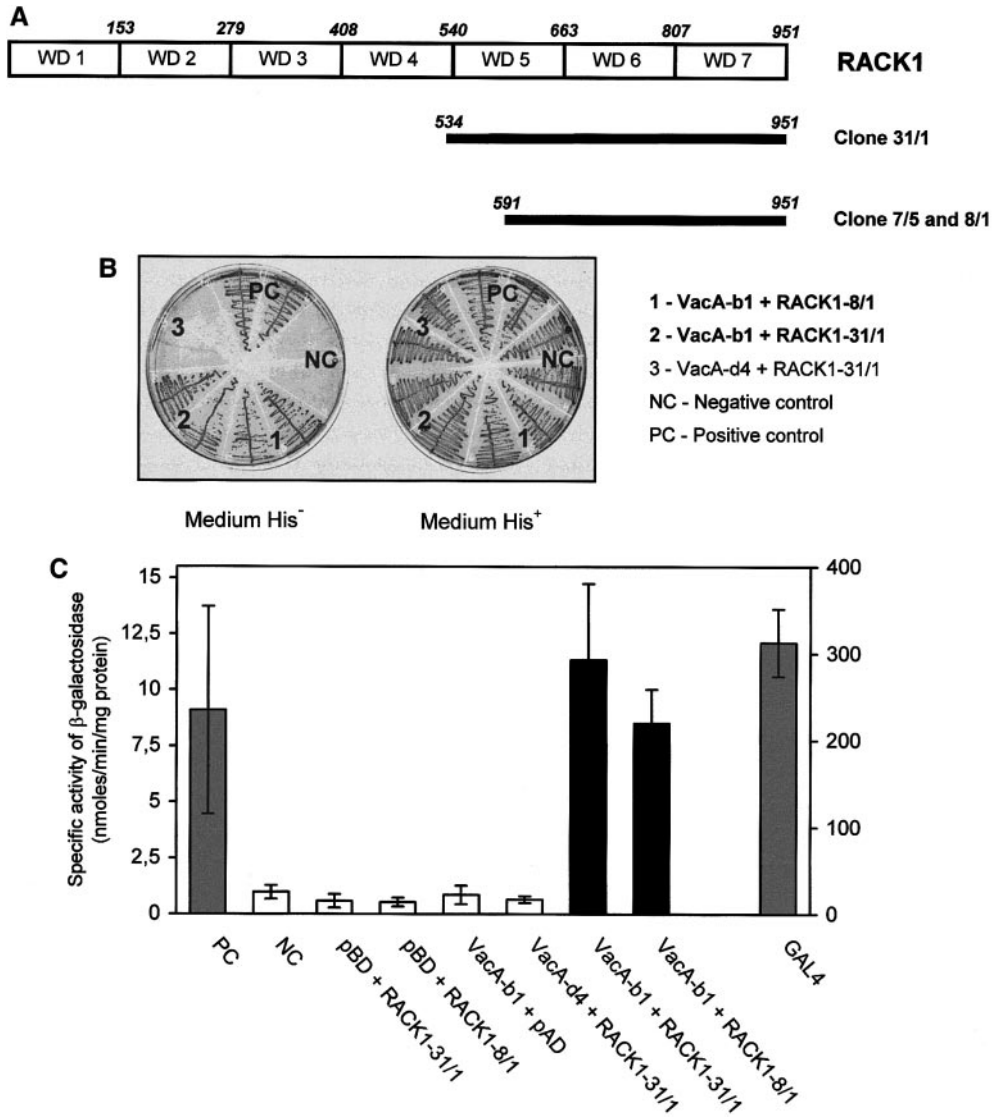


**FIG. 3.** Identification of RACK1 as a protein interacting with VacA-b1 in *S. cerevisiae.* (A) Schematic representation of RACK1 structure and of the clones obtained from the yeast two-hybrid screening. The seven WD40 motifs of RACK1 are outline. The numbers indicate the C-terminal nucleotide of each motif as well as N-terminal position of RACK1 clones isolated by two-hybrid screening. (B) Growth on histidine-free medium. The YRG2 reporter strain was transformed with the plasmids encoding indicated cDNAs and patched onto plates selected for both plasmids (Medium $His^+$) and for protein–protein interaction (Medium $His^-$). For NC, the reporter strain was transformed with pLaminC-BD and pSV40-AD plasmids; for PC the strain was transformed with p53-BD and pSV40-AD plasmids (Stratagene, La Jolla, CA). (C) Quantitative assay of $\beta$-galactosidase activity. The YRG2 strain was transformed with plasmids encoding indicated cDNAs or plasmids without inserts. The NC and PC are as shown above. As a reference, we used plasmid, which contains the entire GAL4 coding region (881 aa). $\beta$-Galactosidase specific activities are calculated as nanomoles of ONPG hydrolized per minute per milligram of protein. The values are averages for four transformants, each assayed in triplicate ($\pm$standard deviations).

### *In Vitro Binding Assay with GST Fusion VacA Protein Fragments*

To confirm the interaction between VacA and RACK1 observed in yeast, we performed *in vitro* binding assays with recombinant proteins expressed in *E. coli* cells. For this purpose, GST fusion proteins VacA-b1 and VacA-S1 (S1 fragment encoded the entire mature toxin; Fig. 1) were immobilized on glutathione–Sepharose (Fig. 4A) and incubated with [$^{35}$S]Met-labeled *in vitro* translated RACK1-8/1 and RACK1-31/1. Bound proteins were detected by SDS-PAGE. As shown in Fig. 4B, both GST-VacA-b1 and GST-VacA-S1 were capable of binding the RACK1-8/1 and RACK1-31/1, in contrast to GST. Two other GST proteins, *H. pylori* urease and human K protein, also did not bind RACK1 *in vitro* (data not shown).

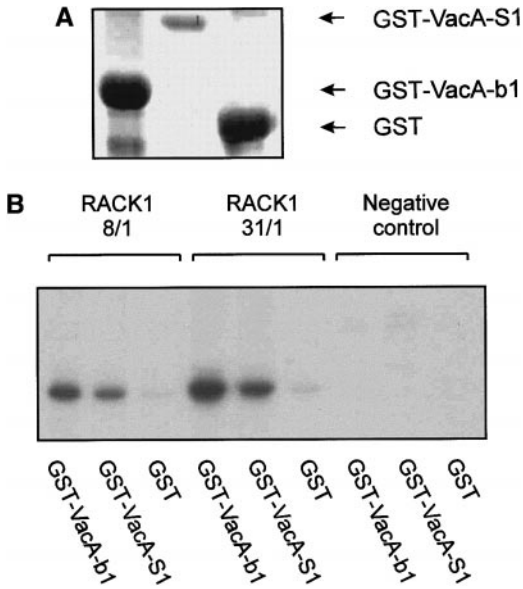


**FIG. 4.** *In vitro* binding of RACK1 protein with VacA in the GST fusion protein binding assay. (A) Coomassie blue staining of SDS–PAGE fractionated GST and GST-VacA protein fragments bound to glutathione–Sepharose beads, and used in each experiment. (B) Autoradiography of [$^{35}$S]Met-labeled RACK1 protein fragments bound to GST-VacA protein fragments. The binding proteins were fractionated by SDS–PAGE and visualized by autoradiography. The luciferase protein encoded by commercial plasmid (Promega Corp., Madison WI) was *in vitro* translated and used as binding negative control.

## DISCUSSION

Our paper reports, for the first time, that RACK1 is a VacA-binding protein. The interaction between VacA and RACK1 was found *in vivo* using yeast two-hybrid system and was confirmed by the *in vitro* pull down assay by the use of *in vitro* translated RACK1 and bacterially expressed GST fusion VacA protein fragments.

The RACK1 interacting VacA-b1 fragment comprises C-terminal portion of p33 VacA subunit and 6 residues from p55 subunit (from 203–350 aa). As was shown by transfection experiments, the minimal intracellular vacuolating domain, necessary for the cytosolic activity of VacA, has been localized between residues 1 and 422 (23, 24), which include the VacA-b1 fragment. Either VacA-c3 or VacA-d4, consisted of p55 subunit fragments, did not interact with RACK1.

RACK1, a 36-kDa homolog of the $\beta$ subunit of G proteins, belongs to a large family of regulatory proteins made up of highly conserved repeating WD40 units (32). WD-repeats represent structural motifs formed by four antiparallel $\beta$-strands and have been implicated in protein-protein interaction. RACK1 is composed of seven such elements (33). The cDNA inserts of two-hybrid positive clones, isolated in this study, code for C-terminal three WD motifs (WD5, WD6 and WD7; Fig. 3A) of RACK1 protein, suggesting that they are sufficient for interaction with VacA. Similarly, the same three WD motifs of RACK1 are involved in binding of integrin $\beta$ subunit cytoplasmic domain (34).

It was shown that RACK1 binds to PKC in the presence of $Ca^{2+}$ and lipid *in vitro* (33) and acts as an intracellular receptor for activated PKC$\beta$II *in vivo* (35). RACK1 is neither a PKC substrate nor an inhibitor. Rather, it increases PKC phosphorylation of substrates presumably by stabilizing the active of the enzyme (33). On the other hand, localization of RACK1 is regulated by PKC activation (35).

In addition to PKC, RACK1 has been reported to interact with several other proteins, including integrin $\beta$-subunit (34), the Src tyrosine kinase (36), the cAMP-specific phosphodiesterase isoform PDE4D5 (37), the Epstein–Barr virus activator protein BZLF1 (38), common $\beta$-chain of the IL-5/IL-3/GM-CSF receptors (39), and pleckstrin homology (PH) domain-containing proteins like dynamin-1 or $\beta$-spectrin (40). However, the mechanisms for these interactions appear to be quit different. Some of them involve phorbol ester-induced conformational changes (33–35), whereas others do not (36–40). Overexpression of RACK1 inhibits the activity of Src tyrosine kinases and the growth of NIH 3T3 cells (36).

It is becoming increasingly apparent that many interactions between molecular partners that are involved transduction and gene expression occur in the context of a novel class of proteins that act as scaffolds. Although, the physiologic role of interactions between RACK1 and most other cellular proteins remains obscure, the multidomain RACK1 might function as a scaffold protein to recruit a variety of proteins into a signaling complex. It was shown that upon activation of PKC, RACK1 and PKC$\beta$II coordinately moved to the same compartment of the cell (35); in addition, both activated PKC and the PH domain of $\beta$-spectrin or dynamin-1 are concomitantly bound to RACK1 (40). Therefore, it might be speculate that RACK1 acts as a shuttling protein that bring PKC in close proximity with its substrate, thus enabling substrate phosphorylation with higher efficiency. RACK1 may also function as PKC-independent membrane-anchored adaptor for non-receptor kinases (Src like kinases), that restrict the activities of associated kinases and phosphatases, bringing them in close proximity with their activators (36).

The functional significance of the association of VacA with RACK1 is unknown. Several studies have shown that *H. pylori* adherence to polarized epithelial cell monolayers resulted in a decrease in *trans*-epithelial electrical resistance (TER), which is linked to the action of acid-activated VacA cytotoxin (41, 42). The *H. pylori*-induced reduction of TER was concomitant with significant increase in the paracellular permeability of

epithelial monolayers and was inhibited by the PKC activator PMA (41–43). Assuming that PKC is involved in epithelial cells tight junction regulation, it is suggested that *H. pylori* may activate some intracellular pathways counteracting the effects of PKC.

Recently, VIP54 protein was identified as VacA-interacting protein in yeast two-hybrid screening of a library derived from HeLa cells (27). It was shown that VIP54 is a novel intermediate filament protein that interacts with vimentin. However, similar to RACK1, it is not known whether VacA-VIP54 interaction is required for toxicity.

It can only be speculated that RACK1 is able to assess multiple inducible factors and allows for their multilateral cross-talk by integrating inputs from different signal pathways. In this context, RACK1 may form the nucleus of a signaling module for VacA intracellular action.

## ACKNOWLEDGMENTS

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