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Interaction with Factor Associated with Neutral Sphingomyelinase Activation, a WD Motif-Containing Protein, Identifies Receptor for Activated C-Kinase 1 as a Novel Component of the Signaling Pathways of the p55 TNF Receptor

Anna Ewgenjewna Tcherkasowa,2* Sabine Adam-Klages,2* Marie-Luise Kruse,† Katja Wiegmann,‡ Sabine Mathieu,* Waldemar Kolanus,§ Martin Krönke,‡ and Dieter Adam3*

Factor associated with neutral sphingomyelinase activation (FAN) represents a p55 TNFR (TNF-R55)-associated protein essential for the activation of neutral sphingomyelinase. By means of the yeast interaction trap system, we have identified the scaffolding protein receptor for activated C-kinase (RACK)1 as an interaction partner of FAN. Mapping studies in yeast revealed that RACK1 is recruited to the C-terminal WD-repeat region of FAN and binds to FAN through a domain located within WD repeats V to VII of RACK1. Our data indicate that binding of both proteins is not mediated by linear motifs but requires folding into a secondary structure, such as the multibladed propeller characteristic of WD-repeat proteins. The interaction of FAN and RACK1 was verified in vitro by glutathione S-transferase-based coprecipitation assays as well as in eukaryotic cells by coinmunoprecipitation experiments. Colocalization studies in transfected cells suggest that TNF-R55 forms a complex with FAN and that this complex recruits RACK1 to the plasma membrane. Furthermore, activation of N-SMase by TNF was strongly enhanced when RACK1, FAN, and a nontoxic TNF-R55 mutant were expressed concurrently, suggesting RACK1 as a modulator of N-SMase activation. Together, these findings implicate RACK1 as a novel component of the signaling pathways of TNF-R55. The Journal of Immunology, 2002, 169: 5161–5170.
as cutaneous barrier repair (17), possible further signaling functions of FAN have largely remained elusive.

Receptors for activated C kinase (RACKs) comprise a group of cytosolic proteins that can bind to activated isoforms of protein kinase C (PKC). It has been suggested that RACKs facilitate the translocation of PKC isoforms to cellular membranes and simultaneously stabilize PKC from premature degradation (18). RACK1 is a 36-kDa protein with homology to the β subunit of heterotrimeric G proteins, that, like FAN, is characterized by the presence of (seven) WD repeats (19), structures which have the potential to fold into multibladed-propeller-like structures (5). The WD repeats in RACK1 are conserved from Chlamydomonas to human (20), indicating that the function of RACK1 was presumably established before the evolutionary divergence of plants and animals. Recent results indicate that RACK1 is a scaffolding protein that is involved in the recruitment, assembly, and regulation of a number of different signaling molecules into membrane-associated complexes. Proteins recruited by RACK1 comprise PKCβII, PKCε, the cytoplasmic tyrosine kinase c-src, the β subunit of integrins, (18, 21–23), as well as a number of other cytoplasmic and viral proteins (24–37). Functionally, RACK1 has been implicated in the development of cardiac hypertrophy and failure, the regulation of cell adhesion, protection of tumor cells from E1A-induced apoptosis, age-associated decline in alveolar macrophage functions, assembly of signaling complexes at the platelet-derived growth factor receptor, integrin-dependent cell migration, negative signaling for peroxide anion generation, and the recruitment of STAT1 to the human type I IFNAR (21, 27, 30, 38–42). Moreover, RACK1 interacts with the pleckstrin homology (PH) domains of several proteins in vitro, indicating that it may aid in colocalizing PKC with its PH domain-containing substrates (43). However, only a subset of these interactions depends upon PKC stimulation (23). In addition, some of the interactions between the proteins listed above and RACK1 appear to be mutually exclusive (22, 27), suggesting that RACK1 forms distinct cellular signaling complexes in response to distinct cellular stimuli.

We have identified RACK1 as an interaction partner of FAN by means of the yeast interaction trap system. RACK1 specifically binds to FAN, as demonstrated by communoprecipitation and GST coprecipitation experiments. Deletion studies in yeast map the interaction domains of both proteins to WD repeats IV to VII of RACK1 (minimally encompassing parts of WD repeat V to WD repeat VII), and to the C-terminal WD-repeat region of FAN. The interaction of FAN and RACK1 appears to be dependent on the folding of the WD repeats into a secondary structure—conceivably the proposed multibladed propeller—because no prominent linear binding motifs were identified in the WD-repeat region of FAN. Confocal laser-scanning microscopy studies suggest that overexpressed FAN and RACK colocalize at the plasma membrane together with TNF-R55. At the functional level, concurrent overexpression of FAN, RACK1, and a noncytotoxic deletion mutant of TNF-R55 strongly enhanced TNF-induced stimulation of N-Smase. Our findings suggest a novel linkage between TNF-R55 and RACK1/PKC-mediated signaling pathways.

Materials and Methods

Yeast interaction trap system

The plex.FAN constructs were generated by cloning restriction fragments of FAN containing amino acids 3–917 (FAN45–917) and 3–547 (FAN45–547) into plex202. Sequencing verified that the inserts were in the correct reading frame. The generation of the Jurkat cDNA library fused to a synthetic activation domain has been described elsewhere (44). The HeLa cDNA expression library was purchased from Clontech Laboratories (Palo Alto, CA). Transformation of the yeast strain EGY48/JK103 (45) with bait constructs and, subsequently, with the library DNA or selected activation domain fusion constructs was performed as described (44). Transformants were grown on Ura ‘His Trp’ glucose plates, before selection for leucine prototrophy on Ura His Trp Leu galactose plates was used to test for positive interaction. Expression of the bait constructs was verified by immunoblotting using a monoclonal anti-Ab (BD Biosciences, Heidelberg, Germany). Testing for β-galactosidase expression was performed on either Ura His Trp galactose X-Gal plates or, for quantification, in a liquid assay. Yeast cells were grown overnight at 30°C in Ura His Trp galactose medium to an OD590 of ~1.8, diluted 1/5 in the same medium and grown at 30°C to an OD600 of 0.5–0.8. Cells were washed, resuspended in Z-buffer (50 mM Na2HPO4/40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, pH 7.0), and lysed by freezing/thawing. The cells were diluted 1/8 in Z buffer containing 0.27% (v/v) 2-ME. The reaction was started by adding 160 µl Z buffer containing 4 mg/ml o-nitrophenyl-β-d-galactoside, incubated at 37°C, and stopped with 0.4 ml of 1 M Na2CO3. After precipitating the cells, the OD420 of the supernatants was determined. β-Galactosidase activity was expressed as units of β-galactosidase activity per mg of cell soluble protein.

In vitro interaction assay

The insert of pG.RACK144–547 coding for amino acids 144 to 317 was expressed as a GST fusion protein using the bacterial expression vector pGEX4T1 (Amersham Biosciences, Freiburg, Germany). Cultures of Escherichia coli XL1-Blue containing pGEX-RACK144–547 were induced with 2 mM isopropyl-β-d-thiogalactoside for 6 h at 37°C. Bacteria were harvested, resuspended in PBS containing 1% (v/v) Triton X-100, and lysed by freeze/thaw cycles. After centrifugation at 12,000 × g for 10 min to remove debris, the supernatant was passed through a glutathione Sepharose 4B column (Amersham Biosciences). The column was washed three times with PBS, and GST fusion protein was eluted by the addition of 50 mM Tris (pH 8.0)-10 mM glutathione according to the manufacturer’s instructions. GST-RACK144–547 fusion protein (15 µg) or GST alone were preincubated with glutathione Sepharose 4B beads for 1 h at 4°C in PBS containing 1% (v/v) Triton X-100 and collected by centrifugation. The precipitates were resuspended in binding buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% (v/v) Nonidet P-40 (NP40), 1 mM EDTA, and 5% (v/v) glycerol) and the entire coding regions of FAN or cytosolic phospholipase A2 (cPLA2) that had been synthesized in vitro using the SP6-coupled TNT Reticulocyte Lysate System (Promega, Madison, WI) and [35S]methionine (Amersham Biosciences) were added. After incubation for 2 h at 4°C, the beads were washed three times in binding buffer containing 300 mM NaCl. Subsequently, the bound proteins were eluted by boiling in SDS sample buffer, resolved by 8% (v/v) SDS-PAGE, and analyzed after autoradiography on a phosphomager.

Mapping of the interaction sites of RACK1 and FAN

For the isolation of a full-length cDNA for RACK1, the insert of pG.RACK144–547, representing the largest of all clones isolated in the yeast interaction trap system, served as a probe to screen a human skeletal muscle cDNA library (lambda ZAP Express; Stratagene, Amsterdam, The Netherlands) by standard methods (46). The cDNA inserts of the isolated phage plaques were excised in vivo, following the instructions of the manufacturer, and sequenced. All clones isolated proved identical, containing the entire coding region of RACK1. Subsequently, the entire coding region of RACK1 (amino acids 1–317) as well as C-terminal fragments coding for amino acids 198–317 and 204–317 were amplified by PCR adding restrictions sites for EcoRI/Sall (RACK1204–317) or EcoRI/Xhol (RACK1204–518 and RACK1204–317). The above fragments were then subcloned into both bait (plex.RACK1204–518) and prey vector pgi4–5 (EcorI/Xhol; Ref. 45) of the yeast interaction trap system. The insert of pG.RACK1204–317 was isolated as an EcoRI/Xhol fragment and subcloned into the EcoRI/Sall sites of plex202. As far as not yet available from the initial yeast interaction trap screens, bait and prey constructs of FAN45–547, FAN45–357, FAN603–917, as well as Apaf-1197–1197 (original construct kindly provided by Dr. X. Wang, Howard Hughes Medical Institute and Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX), were generated by the same procedure as above. All constructs were verified by sequence analysis.

Stappled peptide scanning assay

Cellulose filters containing FAN-derived peptide scanning libraries were purchased from Jerni Bio Tools (Berlin, Germany). Radiolabeled whole cell extracts from Jurkat cells were prepared by incubation of 3 × 106 cells with 2.5 nM in vitro cell-labeling mix (1000 Ci/mmol of L-[35S]methionine and L-[35S]cysteine; Amersham Biosciences) in methionine/cysteine-
free medium for 4 h, followed by two washes in PBS and lysis in HDP (30 mM HEPES pH 7.9, 10% (v/v) glycerol, 7 mM MgCl2, 10 mM KCl, 1 mM DTT, 0.1% (v/v) NP40, 10 μg/ml aprotinin and leupeptin) by freezing/thawing. The cell lysate was incubated on peptide filters that had been preswashed twice in methanol and twice in PBS, in 1× SuperBlock blocking buffer (Pierce, Rockford, IL) in HDP overnight at 4°C with continuous shaking. After five washes at room temperature in NET (150 mM NaCl, 50 mM Tris pH 7.5, 5 mM EDTA, 0.05% (v/v) NP40), filters were air-dried and autoradiographed. Binding of the cell lysates to individual peptides was quantified using a phosphorimager.

**Cell culture**

293 (human embryonic kidney) and COS-1 cells were originally obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in high glucose DMEM supplemented with 10% (v/v) fetal calf serum, 10 mM glutamine, 50 μg/ml penicillin, and 50 μg/ml streptomycin (Invitrogen, Groningen, The Netherlands) in a humidified incubator at 5% (v/v) CO2.

**In vivo interaction assay**

The coding region of RACK1 was amplified by PCR and ligated into pcDNA3.1 MYc-His A (Invitrogen) as an N-terminal fusion to the myc tag of the vector, yielding plasmid pMYC.RACK1-317. For coimmunoprecipitation experiments, 1.8 × 105 293 cells were transiently transfected with 5 μg of pMYC.RACK1-317 or pcDNA3.1 MYc-His A in combination with 5 μg of pFLAG.FAN 317 or pFLAG.CMV2 (6) by the calcium phosphate precipitation method. After 48 h, cells were detached using 2 mM EDTA, lysed in TNE buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% (v/v) NP40, and 2 mM EDTA) containing 10 μg/ml aprotinin/leupeptin, and 3 μg of cellular lysates were precleared with γ-bind-Sepharose (Amersham Biosciences). Immunoprecipitation was performed overnight on ice using 3 μg of anti-FLAG M2 IgG1 mAb (Sigma-Aldrich, Tauferkirchen, Germany) or 1.4 μg of anti-myc IgG1 mAb (Invitrogen) followed by collection of the immunocomplexes by a 1-h incubation with γ-bind-Sepharose and subsequent washing of the immunocomplexes for three times in TNE, once in TNE containing 1 M NaCl, and once more in TNE. The immunoprecipitated proteins were separated on 4–15% gradient gels by SDS-PAGE. For detection of expressed tagged RACK1 and FAN protein in transfected cell lysates, 20 μg of cell protein per lane were resolved by SDS-PAGE. After electrophoretic transfer to nitrocellulose (Whatman, Buckingham, Göttingen, Germany), reactive proteins were detected using anti-myc or anti-FLAG Ab M2 by the ECL detection kit (Amersham Biosciences). Coimmunoprecipitations of endogenous RACK1 and FAN with TNF-R55 were performed essentially as above with the following modifications: untransfected 293 cells were lysed in TNE buffer (50 mM Tris pH 8.0, 140 mM NaCl, and 0.5% (v/v) Brijs 8) supplemented with the Protease Inhibitor Mix Complete (Roche Diagnostics, Mannheim, Germany). Immuno precipitation was performed overnight on ice using 3 μg of anti-FLAG M2 IgG1 mAb (Sigma-Aldrich, Taufkirchen, Germany) or 1 μg of anti-myc IgG1 mAb (Invitrogen) followed by collection of the immunocomplexes by a 1-h incubation with γ-bind-Sepharose and subsequent washing of the immunocomplexes for three times in TNE, once in TNE containing 1 M NaCl, and once more in TNE. The immunoprecipitated proteins were separated on 4–20% (v/v) gradient gels by SDS-PAGE. For detection of expressed tagged RACK1 and FAN protein in transfected cell lysates, 20 μg of cell protein per lane were resolved by SDS-PAGE. After electrophoretic transfer to nitrocellulose (Whatman-Biometra, Göttingen, Germany), reactive proteins were detected using anti-myc and/or anti-FLAG Ab M2 by the ECL detection kit (Amersham Biosciences). Coimmunoprecipitations of endogenous RACK1 and FAN with TNF-R55 were performed essentially as above with the following modifications: untransfected 293 cells were lysed in TNE buffer (50 mM Tris pH 8.0, 140 mM NaCl, and 0.5% (v/v) Brijs 8) supplemented with the Protease Inhibitor Mix Complete (Roche Diagnostics, Mannheim, Germany), 2.7 mg of cellular lysate were immunoprecipitated without preclearing using 3 μg of anti-TNF-R55 htr 9 IgG1 mAb (Bachem, Heidelberg, Germany) or 1 μg of anti-FAN N19 goat polyclonal Ab (Santa Cruz Biotechnology, Heidelberg, Germany) followed by SDS-PAGE, and endogenous RACK1 and FAN proteins present in the immunoprecipitates were detected using either anti-FAN Ab N19 or anti-RACK1 IgM mAb (BD Biosciences).

**Intracellular colocalization studies**

A SalI-EcoRI fragment from the human TNF-R55 encoding amino acids 1–345 was isolated from pADB-TR55 (47) and cloned into the expression vector pEF-BOS (48) yielding pEF-TNF-R55Δ345. COS-1 cells were transiently transfected by electroporation at 280 V/960 μF using 5 μg of pMYC.RACK1-317, pFLAG.FAN 317, and/or pEF-TNF-R55Δ345, and grown to subconfluence on coverslips for 24–48 h before fixation in 2.5% (v/v) paraformaldehyde in PBS for 30 min at room temperature. The coverslips were washed two times in PBS and once for 10 min in TBS to remove the fixative followed by permeabilization and blocking of nonspecific sites with 0.05% (v/v) Saponin and 0.1% (v/v) BSA in TBS containing 0.1% (w/v) glucose for 1 h at room temperature and three washes in TBS for 5 min each. Cells were incubated with primary Abs (anti-myc mouse monoclonal IgG1 (1:100), anti-FLAG mouse monoclonal IgG1 (1:200), affinity-purified anti-FLAG rabbit polyclonal antiserum (1:50; Zymed Laboratories, San Francisco, CA), anti-TNF-R55 goat polyclonal affinity-purified IgG (1:50; R&D Systems, Minneapolis, MN) in TBS for 1 h at 37°C. After washing three times in PBS (5, 10, and 15 min), cells were incubated with secondary Abs (FITC-conjugated donkey anti-rabbit or anti-goat IgG (1:100), rhodamine-conjugated donkey anti-mouse IgG (1:100), CY3-conjugated donkey anti-rabbit IgG (1:350; Dianoia, Hamburg, Germany) for 1 h in TBS at 37°C. Cells were again washed three times in PBS (5, 10, and 15 min) before stained proteins were visualized using a Zeiss LSM 510 confocal laser-scanning microscope (Zeiss, Jena, Germany). Final digital images were processed by increasing contrast of all images by 50% using Adobe Photoshop 4.0 (Adobe Systems, Mountainview, CA).

**Results**

**Yeast interaction trap system**

To identify proteins that directly interact with FAN, we used the yeast interaction trap system. A bait construct containing the full-length FAN cDNA (pEX.FAN317; Fig. 1A) was used to screen a Jurkat cDNA expression library. In 41 cases, the isolated colonies contained inserts that were identical to various proportions of the C terminus of RACK1, with the shortest insert spanning most of WD repeat V to WD repeat VII (Fig. 1B), suggesting that this is the minimal region through which RACK1 interacts with FAN.

**FIGURE 1. Yeast interaction trap screen implicating RACK1 as a FAN-interacting partner. A, Schematic representation (drawn to scale) of the FAN protein and of the bait constructs FAN317 and FAN547. The conserved GRAM, BEACH, and WD homology regions of FAN are indicated. The constructs FAN317 and FAN547 that was used in the mapping of the interaction sites of FAN and RACK1 is shown as well. B, Amino acid sequence of the human recombinant RACK1 protein and of the bait constructs FAN 317, or pFLAG.FAN317, and/or pEF-TNF-R55Δ345, either individually or as a combination of all three constructs, by the calcium phosphate precipitation method. After 48 h, cells were treated in triplicate in 0.5 ml medium with 100 ng/ml human recombinant TNF (a gift from Dr. G. Adolf, Bender Research Institute, Vienna, Austria) for 0–5 min. Cells were homogenized as described (49), except that 0.5% (w/v) 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS) was added to the buffer. Radioactive phosphocholine produced from [(N-methyl-14C)-phosphatidylcholine (labeled in the choline moiety, CFA566; Amersham Biosciences) was identified by TLC and routinely determined in the aqueous phase by scintillation counting.
RACK1 interacts with FAN in vitro

The association of FAN and RACK1 was further investigated by in vitro binding experiments in a cell-free system. For this purpose, the insert of pJG.RACK144–317 was expressed as a GST fusion protein and tested for its ability to bind to [35S]methionine-labeled, in-vitro-translated, full-length FAN or the unrelated cPLA2. The cPLA2 negative control showed the same pattern of bands with GST alone and with GST-RACK1198–317, indicating that no specific binding of RACK1 to cPLA2 occurred (Fig. 2, lanes 2 and 4). In contrast, FAN bound to GST-RACK1198–317 (Fig. 2, lane 3, asterisk) but not to GST alone (lane 1).

Mapping of the interaction sites of RACK1 and FAN

Bait and prey constructs for RACK11–317 (the entire coding region), RACK1144–317 (the largest clone isolated in the above screen), RACK1198–317 (the smallest clone isolated), and RACK1204–317 (Fig. 3), as well as for FAN3–917, FAN5–547, FAN703–917 (containing WD repeats II to V at the C-terminus of FAN, Fig. 1A), and Apaf-1577–1182 (containing the C-terminal WD repeats I to XII of Apaf-1) were generated as described under Materials and Methods. Combinations of bait and prey constructs were cotransformed into yeast, as outlined in Table I, and tested for β-galactosidase expression. In controls for nonspecific interaction with prey vector pJG4-5 without insert, all bait constructs were negative (data not shown). In line with the results from the previous screens, RACK1144–317 strongly interacted with full-length FAN regardless of which construct was used as bait or prey. This RACK1 construct also interacted as both bait and prey with FAN703–917 containing only the C-terminal WD repeats, confirming that both proteins interact through their C-terminal WD repeats. The RACK1144–317 bait construct interacted with a RACK1144–317 prey construct (self-association); however, it did not show any binding to the WD-repeat-containing C terminus of Apaf-1. Therefore, the interaction of RACK1144–317 and FAN is specific and not due to a general stickiness of the WD-repeat regions of both proteins.

RACK1198–317, the smallest FAN-interacting clone isolated in the previous screens, showed a weaker interaction with full-length FAN as bait but no interaction with full-length FAN as prey. We have observed this nonreciprocal behavior especially in weak interactions, and we attribute it to intrinsic differences in the steric interaction of both partners when bait and prey are switched. Nevertheless, the fact that this clone still interacted with FAN3–917 as bait in the above experiment indicated that this clone contains the minimal domain required for FAN interaction. When tested for interaction with FAN3–917 containing only the C-terminal WD repeats of FAN, RACK1198–317 still showed a strong binding. Like RACK1144–317, RACK1198–317 showed a pronounced self-association.

In line with the assumption that RACK1198–317 contained the minimal FAN-binding site, the even smaller construct RACK1204–317 did not bind to full-length FAN anymore in any bait/prey combination. Also, compared with RACK1198–317, the interaction with the WD-repeat coding construct FAN703–917 was strongly reduced. However, RACK1204–317 was still able to strongly associate with itself, indicating that this core fragment retained general binding activity but that specific binding to FAN required additional determinants.

When the entire RACK1 protein was used as bait or prey, we found interaction with the C-terminal WD repeats of FAN703–917 but, surprisingly, we could not detect an interaction with full-length FAN. However, full-length FAN also did not show an association with TNF-R55 in these experiments (as measured by β-galactosidase expression; data not shown), although this association has been clearly shown in vivo (6). Moreover, it has been demonstrated that the interaction of RACK1 with the β subunit of integrins or the HIV-1 Nef protein is detectable in yeast with C-terminal RACK1 constructs containing WD repeats V to VII, but not with full-length RACK1 (23, 33). These results are most likely indicative that RACK1 interacts with FAN in vitro binding experiments in a cell-free system.
Table I. Mapping of the interaction sites of FAN and RACK1

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<th>RACK1&lt;sub&gt;198-317&lt;/sub&gt;</th>
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due to limitations of the yeast interaction trap system when the expressed interaction partners become too large to allow the fused DNA-binding and activation domains to drive β-galactosidase expression. In line with this assumption, neither full-length RACK1 nor FAN showed self-association in these assays.

All RACK1 constructs showed no or only very weak interaction with FAN<sub>3-547</sub> harboring the putative N-terminal effector domain. In summary, the above experiments indicate that the interaction of RACK1 with FAN is mediated by a core region of RACK1 that comprises WD repeats IV to VII and that minimally must encompass parts of WD repeat V (C-terminal of amino acid 198) as well as the WD repeats VI and VII (Fig. 3).

In an independent approach, we wanted to determine whether FAN contained further potential protein-binding sites. Similar to a previous experiment that had identified the binding motif for FAN within TNF-R55 (6), we used a cellulose-bound peptide scanning library staggered by three amino acids each, containing overlapping 13-mer peptides of the entire FAN protein (Fig. 4A). Jurkat cells were metabolically labeled, and total cellular extracts were assayed for binding to the staggered peptides. Within the putative effector domain of FAN, regions of significant binding spanned peptides 14–18, 57–60, 79–80, and 189–191. However, none of the peptides contained in these regions is localized entirely within the GRAM or BEACH domain of FAN. The only peptides from these domains that bound cellular proteins significantly over background were peptides 121 and 153–154, both located in the BEACH domain (Fig. 4B). Apparently, the GRAM and BEACH domains of FAN do not contain extended linear protein-binding motifs detectable by the staggered peptide scanning assay. Nevertheless, the peptide stretches detected outside of both domains may mark the binding sites of yet-unidentified proteins potentially participating in the signal transduction pathways of FAN. Within the WD repeats of FAN, only peptide 288 displayed significant binding to the labeled extracts (Fig. 4B). This was somewhat surprising, because it could be expected that RACK1 or other WD-repeat binding proteins present in the labeled extracts should produce a “footprint” of labeled peptides in this assay. Apparently, the WD repeats of FAN must be folded into the suggested multibladed propeller secondary structure for interaction with other proteins such as RACK1. Because this secondary structure is not represented adequately by linear binding motifs, the interaction of FAN and RACK1 is apparently not detected by the staggered peptide scanning assay.

Coimmunoprecipitation of RACK1 and FAN

To elucidate whether full-length RACK1 and FAN were able to interact in intact cells, 293 cells were transiently transfected with a myc-tagged version of RACK1 (pMYC.RACK<sub>1-317</sub>) in combination with a FLAG-tagged full-length FAN construct (pFLAG.FAN<sub>3-917</sub>; Ref. 6). For control, cells were additionally transfected with pMYC.RACK<sub>1-317</sub> or pFLAG.FAN<sub>3-917</sub>, in combination with the parental vectors pFLAG.CMV2 or pcDNA3.1<sup>myc</sup>/Myc-His A, and finally with both empty vectors alone. The expression of the transfected fusion proteins was confirmed in Western blots with anti-myc and anti-FLAG Abs (Fig. 5, A and B; lower panels). In parallel, identical amounts of cellular lysates were immunoprecipitated using anti-FLAG Ab and coimmunoprecipitating RACK1 was detected in Western blots with anti-myc Ab. As shown in Fig. 5A (upper panel), RACK1 was present only in FAN immunoprecipitates (lane 2) but not in control immunoprecipitates (lanes 1, 3, and 4). In reverse experiments, RACK1 was immunoprecipitated with anti-myc Ab and coimmunoprecipitating FAN was detected in Western blots with anti-FLAG Ab. A band corresponding to the exact size of FAN was detected specifically in RACK1 immunoprecipitates (Fig. 5B, upper panel, lane 2). Similar results were obtained when RACK1 was immunoprecipitated with a mAb directed against RACK1 itself (data not shown). Because the interaction of overexpressed proteins has to be interpreted with caution, we additionally examined whether the endogenous RACK1 and FAN proteins could be detected in a complex with TNF-R55. For this purpose, TNF-R55 was immunoprecipitated from nontransfected 293 cells, and the presence of endogenous FAN and RACK1 in the immunocomplexes was analyzed with Abs against the endogenous proteins. In the case of FAN, a very faint band was detected (Fig. 5C, lane 2). This band exactly corresponds to the predicted size of the FAN protein, and also exactly corresponds to the more prominent band detected in a control immunoprecipitation of the total endogenous FAN protein followed by Western blot with the same Ab (Fig. 5C, lane 1). Similarly, a band of the predicted size of RACK1 was detected in the TNF-R55 immunoprecipitates by the Ab against endogenous RACK1 (Fig. 5D). Identical results (with proportionally stronger bands) were obtained when 293 cells transfected with pMYC.RACK<sub>1-317</sub> or pFLAG. FAN<sub>3-917</sub> were analyzed (data not shown). In summary, these data suggest that not only overexpressed but also endogenous FAN and RACK1 are present in a complex with TNF-R55 in intact cells.

Intracellular colocalization of RACK1 with FAN

The intracellular distribution of FAN and RACK1 was investigated by indirect immunofluorescence staining in combination with confocal laser-scanning microscopy. Because the FAN Ab used in the coimmunoprecipitation experiments did not recognize endogenous FAN with sufficient specificity for immunofluorescence analyses (data not shown), we analyzed COS-1 cells that were transiently transfected with FLAG-tagged FAN (pFLAG. FAN<sub>3-917</sub>) in combination with myc-tagged RACK1 (pMYC. RACK<sub>1-317</sub>) and detected the tagged proteins with anti-FLAG and anti-myc Abs. Both FAN (Fig. 6A, green) and—in line with previous reports (27, 39, 42, 50)—RACK1 (Fig. 6B, red) were detected in the cytoplasm, but not in the nucleus of the cells. The overlay image shows some overlap in the localizations of FAN and
FIGURE 4. Mapping of linear protein binding sites on the FAN protein. A, 13-mer peptides from the FAN protein sequence staggered by three amino acids bound to cellulose membranes that were used in the staggered peptides scanning assay. B, Protein binding domains of FAN. [35 S]methionine-labeled whole cell extract from Jurkat cells was assayed for binding to the FAN-derived peptide scanning library. Bound radioactivity was visualized by autoradiography and quantified using a phosphoimager. The conserved FAN homology domains GRAM and BEACH are indicated, and the WD repeats of FAN are marked by Roman numerals.
RACK1 (Fig. 6, yellow). However, the association of FAN and RACK1 became much more prominent in experiments in which we additionally transfected a truncated form of TNF-R55 (TNF-R55/H9004345). This mutant contains a C-terminal deletion that destroys the death domain, thereby eliminating the cytotoxic effects of an overexpressed TNF-R55, but still carries a functional NSD that permits binding of FAN (4, 49). In the presence of TNF-R55/H9004345 (Fig. 6, green), FAN (Fig. 6, blue) and RACK1 (Fig. 6, red) showed a translocation from the cytoplasm to the plasma membrane, and a pronounced colocalization of all three proteins at the plasma membrane was detected in the overlay of the individual detection channels (Fig. 6, white). In experiments in which only pFLAG.FAN3–917 and pEF.TNF-R55/H9004345 were transfected into COS-1 cells, an identical colocalization of FAN and TNF-R55 at the plasma membrane could be observed as yellow coloring (Fig. 6, white). However, when COS-1 cells were transfected with pEF.TNF-R55Δ345 in combination with pMYC.RACK1–317, RACK1 did not colocalize with TNF-R55 but stayed distributed throughout the cytoplasm (Fig. 6, green). This lack of colocalization did not change after treatment of cells with TNF (although TNF clearly induced internalization of TNF-R55; Fig. 6, N–P), indicating that the endogenous FAN present in these cells is most likely not abundant enough to induce a detectable colocalization of the overexpressed TNF-R55 and RACK1 molecules, regardless of the presence of TNF. Although experiments with overexpressed proteins have to be interpreted with care, these results suggest that the association of FAN and RACK1 is apparently enhanced by the membrane receptor TNF-R55. As shown above, TNF-R55 most likely first forms a complex with FAN at the plasma membrane (Fig. 6, white). This complex may then recruit RACK1 more efficiently (Fig. 6, red) than FAN alone recruits (Fig. 6, blue). As TNF-R55 is expressed only in very low receptor numbers due to the intrinsic cytotoxicity of its cytoplasmic domain (51), the TNF-R55-FAN-RACK1 complexes might not become visible in cells expressing only the endogenous TNF-R55 (Fig. 6, green).

**RACK1 modulates the activation of N-SMase**

Because FAN is required for the activation of N-SMase by TNF, we investigated whether RACK1 is also part of the signaling pathway leading from TNF-R55 to N-SMase. 293 cells were transiently transfected with control vector pcDNA3.1, pFLAG.FAN3–917, pMYC.RACK1–317 or pEF.TNF-R55Δ345 either as individual plasmids or as a combination of all three constructs. As shown in Fig. 7 and in line with previous results (6), transfection of pFLAG.FAN3–917 enhanced the activity of N-SMase after TNF treatment. In contrast, transfection of pMYC.RACK1–317 consistently resulted in a diminished stimulation of

**FIGURE 5.** RACK1 and FAN associate in vivo. A, 293 cells were transiently transfected with expression constructs (5 μg each) for myc-tagged RACK1, FLAG-tagged FAN, and control vectors without insert. **Upper panel,** Coimmunoprecipitation of RACK1 with FAN. Cell lysate (3 mg) was incubated with anti-FLAG Ab M2 to immunoprecipitate FAN and blotted with anti-myc Ab to detect coimmunoprecipitating RACK1. **Lower panel,** Western Blot for expression of FAN and RACK1 in 293 cells. Protein (20 μg per lane) was loaded and analyzed with a combination of anti-FLAG and anti-myc Ab. B, 293 cells were transfected as in A. **Upper panel,** reverse coimmunoprecipitation of FAN with RACK1. Cell lysate (2 mg) was incubated with anti-myc Ab to immunoprecipitate RACK1 and blotted with anti-FLAG Ab M2 to detect coimmunoprecipitating FAN. **Lower panel,** Western Blot for expression of FAN and RACK as in A. C and D, Endogenous FAN and RACK are present in a complex with TNF-R55. The endogenous TNF-R55 was immunoprecipitated from 2.7 mg of 293-cell lysate with the monoclonal anti-TNF-R55 Ab htr-9 and blotted with anti-FAN Ab to detect coimmunoprecipitating endogenous FAN (C, lane 2, arrow) or with anti-RACK1 Ab to detect endogenous RACK1 present in the complex (D). For control, total endogenous FAN was immunoprecipitated and blotted using anti-FAN Ab (C, lane 1). The positions of molecular mass markers (in kilodaltons) are indicated on the left.
N-SMase by TNF. Overexpression of TNF-R55Δ345 did not significantly alter the response of N-SMase toward TNF, confirming that TNF-dependent N-SMase activity is not elevated by stable expression of high numbers of noncytotoxic TNF-R55 deletion mutants (4). Similar to the colocalization experiments where combined expression of all three proteins showed the most pronounced colocalization, the simultaneous transfection of pFLAG.FAN$_{3-917}$, pMyc.RACK1$_{3-317}$, and pEF.TNF-R55/H9004$_{345}$ into 293 cells resulted in the strongest increase in the sensitivity of N-SMase toward TNF, supporting the notion that a complex of TNF-R55, FAN, and RACK1 may be most efficient in stimulating N-SMase. Taken together, these results suggest that RACK1 has a modulating function in N-SMase activation, and implicate RACK1 as a novel component in the signaling pathways of TNF-R55.

Discussion

FAN and RACK1 both represent members of the evolutionarily conserved WD-repeat family of proteins. This protein family contributes to cellular functions as diverse as mRNA processing, transcriptional regulation, assembly of the cytoskeleton, control of cell division, and vesicular trafficking (5). WD-repeat proteins that have been implicated in signal transduction include the $\beta$-chains of heterotrimeric G proteins (52), the phospholipase A$_2$-activator protein PLAP (53), the regulatory subunit of phosphatase 2A (54), as well as FAN and RACK1 themselves (6, 19).

Here, we report the isolation of RACK1 as a FAN-binding protein and present evidence that RACK1 modulates activation of N-SMase by TNF. The interaction of RACK1 and FAN was established by...
multiple independent methods, including 1) the yeast interaction trap system, 2) the binding of in-vitro-translated FAN to a bacterially expressed GST-RACK1 fusion protein, 3) communoprecipitation experiments of overexpressed as well as endogenous proteins, and 4) colocalization studies of FAN, RACK1, and TNF-R55 in whole cells. Our results indicate that the interaction of FAN and RACK1 most likely requires folding of the WD repeats into a secondary structure—possibly the proposed multibladed-propeller structure—because no prominent linear protein-binding motifs were identified within the WD-repeat region of FAN. Deletion studies confirm that binding of FAN and RACK1 requires a crucial region in the C-terminal WD-repeat region of FAN. RACK1, in turn, interacts with FAN through a region comprising at least parts of WD repeat V to WD repeat VI. Because this region is sufficient for binding, it is conceivable that folding of RACK1 into a complete seven-bladed-propeller structure is not essential for its association with FAN. The very same region of RACK1 has also been implicated in its binding to the integrin \( \beta \) subunit and to the HIV-1 Nef protein as well as to the cAMP-specific phosphodiesterase isofrom PDE4D5 (23, 33, 55). Obviously, this region constitutes an interface through which RACK1 can interact with various different proteins.

We have shown that, upon overexpression as tagged proteins, full-length RACK1 communoprecipitates with full-length FAN and vice versa, demonstrating that the two proteins form a complex in intact cells. However, because RACK1 can bind to many different proteins, the significance of its association with FAN in artificial systems, such as yeast, or upon overexpression had to be verified by additional experiments. By immunoprecipitating the endogenous TNF-R55 from nontransfected cells, we were able to demonstrate the presence of endogenous FAN and RACK1 in the immunocomplexes by Western blots. Despite the limited specificity of the currently available reagents against endogenous FAN and RACK1, these experiments provide unequivocal evidence that both endogenous FAN and RACK1 do form a complex with TNF-R55 in untransfected cells, further strengthening the notion of RACK1 as a mediator of TNF-R55-induced signals. However, the question remains whether RACK1 precipitates by direct interaction with TNF-R55 or indirectly via endogenous FAN that is bound to TNF-R55. Because we had also isolated a fragment of RACK1 during the yeast interaction trap experiments in which FAN was identified as an interactor of TNF-R55 (our unpublished observation), we currently cannot exclude the possibility that RACK1 and TNF-R55 are able to interact directly to some extent, at least in the yeast system. In support of these findings, our colocalization data suggest that FAN and TNF-R55 form a complex at the plasma membrane, to which RACK1 colocalizes more efficiently than to either FAN or TNF-R55 alone. Therefore, we propose that TNF-R55, FAN, and RACK1 may be able to form a trimolecular complex in which all three proteins contain binding sites for the other two partners, similar to a complex between PKC, RACK1, and PH domain-containing proteins described by Rodriguez et al. (43). However, the formal proof for the existence of a trimolecular complex not only of overexpressed but also of endogenous TNF-R55, FAN, and RACK1 will have to await the development of more specific reagents.

Definitive evidence for a role of RACK1 in the signaling machinery of TNF-R55 is provided by our observation that RACK1 modulates the sensitivity of N-SMase toward TNF. The inhibitory effect of overexpressed RACK1 on the activation of N-SMase by TNF suggests that an excess of RACK1 may interfere by binding to endogenous FAN, thereby making it unavailable for the transduction of signals from TNF-R55. Notably, overexpression of TNF-R55Δ345 alone did not significantly alter activation of N-SMase by TNF, while overexpression of FAN enhanced the response of N-SMase to TNF. Thus, it appears that, in normal cells, the response of N-SMase to TNF seems to be limited by downstream components of the signaling pathway, such as FAN. RACK1 apparently also belongs to these components, because the simultaneous overexpression of TNF-R55Δ345, FAN, and RACK1 increased the responsiveness of N-SMase to TNF in a substantial manner when compared with any individual transfection. In summary, these results clearly define RACK1 as a functional component of the signaling pathway from TNF-R55 to N-SMase.

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References


RACK1 IS A MEDIATOR OF TNF SIGNAL TRANSDUCTION


