Biochemical and Biophysical Research Communications **259,** 113–120 (1999)
Article ID bbrc.1999.0727, available online at http://www.idealibrary.com on IDEAL®

Sequence Analysis Identifies a Ras-Associating (RA)-like Domain in the N-Termini of Band 4.1/JEF Domains and in the Grb7/10/14 Adapter Family

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Received April 6, 1999

RA (RalGEF/AF6 or Ras-associating) domains are found in a wide variety of proteins, several of which are known to be Ras-GTP effectors. The three dimensional structure of the RA domain has been experimentally determined in Ral-guanine nucleotide exchange factor (Ral-GEF) and found to be similar to that of the Ras-binding domain of c-Raf1, in spite of a very low level of sequence identity. Using various approaches of sequence analysis, including automatic procedures such as BLAST2, profilescan, and hidden Markov models (HMM), as well as the bidimensional hydrophobic cluster analysis (HCA), here we found that a region with a similar structure is also present at the N-terminus of the band 4.1/JEF domain of KIAA0316 (a human cDNA open reading frame) and H09G03.2 (a related protein sequence predicted from *C. elegans* genome cloning), as well as in a particular class of adapter proteins including Grb7, Grb10, Grb14, MIG-10, and PRP48. Although the structural conservation of this motif does not necessarily imply a conservation of its ability to bind small GTPases of the Ras superfamily, several proteins with a band 4.1/JEF domain and adapters of the Grb7 group have close functional relationships with such small GTPases. Thus, our finding raises the intriguing possibility of a direct interaction between members of these two groups of proteins and Ras-like GTP-binding proteins.

Small GTP-binding proteins (G-proteins) of the Ras superfamily play a critical role in signal transduction and control a variety of major biological functions including intracellular trafficking and cell shape, growth and differentiation. These "switch molecules" cycle between GDP-bound inactive and GTP-bound active forms (1). This process is regulated by several classes of proteins including guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs) (2). To understand the physiological function of small GTPases it is critical to identify their downstream targets, of which only a limited number is known. Well-characterised examples include serine threonine kinases such as Raf, Rho-kinase or p21-activated kinase PAK (3-5). In fact, there is no sharp boundary between the regulators and effectors of small GTPases since, in some cases, the same proteins can have both functions. Several conserved domains of interaction with Ras superfamily GTPases have been defined and their three-dimensional structures have been experimentally determined (6-12). One of these is RA (Ral GDS/AF6 or Ras-Associating), a conserved domain found in a variety of proteins, several of which (e.g. Ral GDS (Guanine nucleotide Dissociation Stimulator) and AF6) are known to be RasGTP effectors (13). Interestingly, the structure of a representative member of this family, RalGDS, also named RalGEF (Guanine nucleotide Exchange Factor) (14-16), is very similar to the Ras-binding domain of the protein kinase c-Raf (17), although these two effectors of RasGTP display little sequence identity (13%). These two similar structures belong to the ubiquitin α/β roll superfold (18).

Here we present evidence, based on sequence analysis, for the presence of RA-like domains in two important classes of proteins. First, this domain could be identified in the amino-terminal region of representative examples of a larger conserved domain, the band 4.1/JEF (JAK, ERM, FAK) domain. This domain represents a recent extension of the well-characterised

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0006-291X/99 $30.00

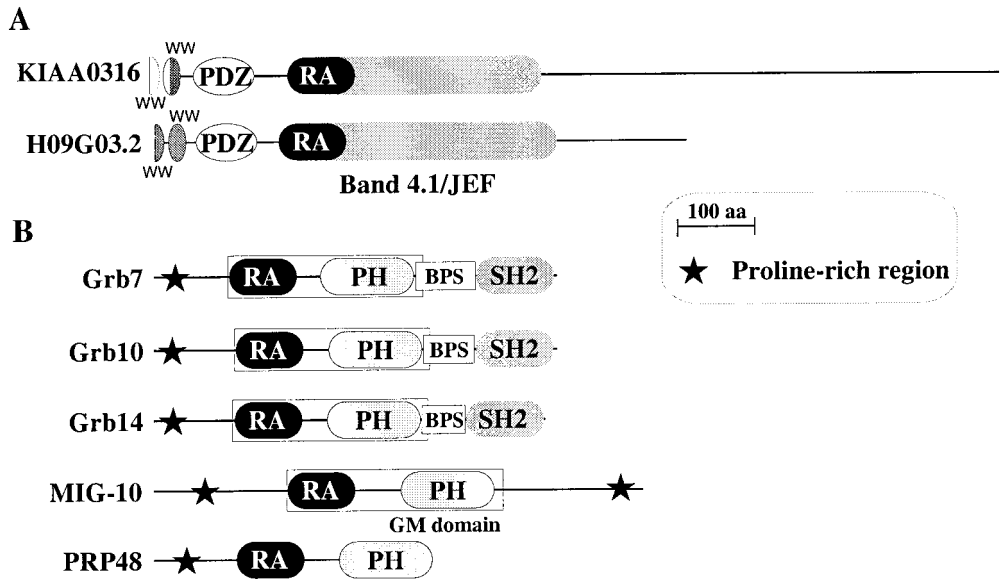

FIG. 1. Schematic domain organisation of human KIAA0316 and *C. elegans* H09G03.2, two proteins that possess a band 4.1/JEF domain (A), as well as of members of the Grb7/Grb10/Grb14 family (B). The protein sequences are drawn to scale. The 5′ untranslated region of human KIAA0316 codes for amino acids sharing high similarity with the very N-terminus of *C. elegans* H09G03.2 (50% identity, not shaded on the KIAA0316 sequence). GenBank or SwissProt accession numbers are those described in Figure 3. Domains are designated as follows (39): **SH2,** Src-Homology 2, **PH,** Pleckstrin Homology, **BPS,** Between Pleckstrin Homology and SH2 (62), also named IPS (Insert between the PH and SH2 domains (61); **Band 4.1/JEF,** Band 4.1 / JAK, ERM, FAK (19); **WW,** WW domain; **PDZ,** PSD-95/Dlg/Zo-1.

band 4.1 domain (19, 20). The band 4.1 domain, also referred as FERM (Four point one/Ezrin/Radixin/Moesin) (21), was originally described as the N-terminal chymotrypsin-resistant region of erythrocyte protein band 4.1 which is able to bind glycophorin C (22, 23). It was found in a large variety of proteins, including the related ezrin, radixin and moesin (ERM), talin, unconventional myosins and several protein tyrosine phosphatases (21, 24, 25). Band 4.1 domains share the ability to interact with the membrane-proximal region of the C-terminal cytoplasmic tail of proteins with a single transmembrane segment (21, 24, 25). Recent work has shown the presence of a similar domain in the N-terminal region of two groups of tyrosine kinases, the FAKs (Focal Adhesion Kinases) (19, 20, 26) and the JAKs (JAnus Kinases) (19, 20) in which they also appear to mediate interaction with transmembrane proteins. Second, we also show that RA-like domains are present in adapter proteins of the Grb7/10/14 family, which bind, through their C-terminal Src-Homology 2 (SH2) domain, phosphotyrosine-containing sequences on a variety of activated tyrosine kinase receptors, including epidermal growth factor receptor (27), insulin receptor (28-32) and insulin-like growth factor (29, 30, 33). Although there is no experimental evidence that these domains are functional in terms of binding Ras-superfamily members, our observations allow to propose several working hypothesis that will be discussed regarding the physiology of these various proteins.

MATERIALS AND METHODS

Searches in the non-redundant database (NR; 356,412 sequences) were performed using BLAST2 (version 2.0.8, scoring matrix Blosum62) and PSI-BLAST programs running at the National Center for Biological Information (NCBI, USA) (34). Hidden Markov Model (HMM) searches were carried out using the HMMER package (35). Profilescan was run at the ISREC server (http://www.isrec.isb-sib.ch/software/PFSCAN_form.html). Guidelines to the use of bidimensional Hydrophobic Cluster Analysis (HCA) are described elsewhere (36, 37). HCA combines sequence comparison with secondary structure predictions and is particularly efficient at low levels of sequence identity (around 15% sequence identity). The HCA score is proportional to the number of hydrophobic amino acids which are topologically conserved (often not chemically identical), and therefore reflects the degree of conservation of the hydrophobic core (38). High HCA scores are associated with low root mean squares values between three-dimensional structures (38).

RESULTS

In the course of our attempts to identify new members of the band 4.1/JEF domain family, we searched the NCBI NR database using the BLAST2 program. We identified an hypothetical protein from *C. elegans*, the putative product of the gene H09G03.2, as a close homologue of the human open reading frame KIAA0316 (29% identity over 539 amino acids, BLAST2 E-value = 3×10^{-65}). Both proteins have a PSD-95/Dlg/Zo-1 (PDZ) domain followed by a band 4.1/JEF domain (Figure 1A). In addition, a complete WW domain, a small module characterised by the presence of two highly conserved tryptophanes (39), is found up-

stream from the PDZ domain in *C. elegans* H09G03.2 (Figure 1A). The N-terminal sequence of human KIAA0316, as retrieved from Genbank, contains only an incomplete WW domain. However, the 5′ supposedly untranslated region codes for amino acid residues that are conserved in H09G03.2 and correspond to the WW domain. This suggests that there may be a functional initiation codon upstream from the proposed ATG that may have been missed due to a phase shift following a sequencing error. Another incomplete WW domain is also found in the very N-terminal sequence of *C. elegans* H09G03.2, which appears also conserved in the 5′ untranslated region of human KIAA0316 (Figure 1A).

We then searched the NR database using an iterative strategy with the *C. elegans* H09G03.2 band 4.1/JEF domain as a seed. A BLAST2 hit was observed, just below the significance threshold (E-value = 14; 24% identity over 90 amino acids) with the human Grb7 protein (aa 101 to 191), which belongs to an adapter-protein family containing the mammalian Grb7, Grb10 and Grb14 proteins as well as *C. elegans* MIG10 (see discussion). All these proteins share a conserved central domain (the GM domain, for Grb and MIG10) of approximately 300 amino acids containing a pleckstrin homology (PH) domain (Figure 1B). In all cases, the GM domains are flanked by a N-terminal proline-rich region. Grb7, Grb10 and Grb14 possess, in addition, a C-terminal SH2 domain (Figure 1B). The region of similarity with *C. elegans* H09G03.2 corresponds to an as yet uncharacterised region located immediately N-terminal to the PH domain in the GM domain. A profilescan run at the ISREC server using the Grb7 sequence as query, significantly matched the profile corresponding to the RA_DOMAIN (PS50200; Nscore = 24.24). Similar results were obtained with all the members of the Grb7 family (Nscore ranging between 16.04 and 22.66 relative to the PS50200 profile), confirming the existence of a RA-like domain in all these proteins. Conversely, a Hidden Markov Model (HMM) search using the multiple alignment of the RA family (Pfam accession number PF00788) also significantly detected the Grb7/Grb10/Grb14 family (scores of 18.30 and 23.66 for Grb7 and Grb14, respectively). A profilescan run independently with *C. elegans* H09G03.2 and human KIAA0316 sequences, also results in significant matches with the RA_DOMAIN profile (Nscore of 10.23 and 9.55, respectively).

Comparison of the HCA plot of the RA domain of RalGDS, whose three-dimensional structure is known, with those of the RA-like domains of Grb7, H09G03.2 and KIAA0316 shows the presence in all these proteins of conserved hydrophobic clusters and key residues, further supporting the existence of similar secondary structures (Figure 2). HCA scores above 60% (e.g. 62%, 61% and 62% for the comparisons of RalGDS with human Grb7, human KIAA0316 and *C. elegans* H09G03.2, respectively) are comparable to those generally observed between related structures with divergent sequences (38). Interestingly, the conserved residues, most of which are closely associated with hydrophobic clusters, mainly correspond to the few residues that are conserved between RalGDS and c-Raf (Figure 2). The corresponding 1D alignment of the RA-like domain of all these proteins is shown on Figure 3.

Thus, we unambiguously detected RA-like domains in all members of the Grb7/10/14 family and related proteins. We also identified this domain in two proteins with a band 4.1/JEF domain. In both families, the relatedness to the RA family is strongly supported by significant values of the comparison with the profile derived from the multiple alignment of the RA family members. In proteins containing a band 4.1/JEF domain, the N-terminal limit of the RA-like domain exactly corresponds to that of the band 4.1/JEF domain in which the RA-like domain is included. The length of the RA-like domain is approximately 90-100 amino acids and it includes conserved blocks 1 to 6 of the band 4.1/JEF domain (i.e., approximately its N-terminal third) (19). The length of the sequences linking the conserved blocks within this part of the band 4.1/JEF domain is much less variable than in the rest of the domain, suggesting stronger structural and/or functional constraints in this region. Interestingly, in the JAK family, the RA-like domain is separated from downstream sequences by a clear hydrophilic hinge and corresponds exactly to the JAK Homology 7 (JH7) region (mouse JAK2: amino acids 37 to 123), one of the seven regions of the JAK family defined on the basis of sequence similarities among the JAK family members (40).

DISCUSSION

In the present study we provide evidence for the presence of a RA-like domain in proteins of the Grb7/10/14 family and within the band 4.1/JEF domain. Although in the latter case the RA-like domain could be identified at a statistically significant level in only two members of the family, the strong conservation of the band 4.1/JEF domain suggests that the N-terminal third of this domain has a RA-like structure. It should be kept in mind that the three-dimensional structure of the RA domain was experimentally demonstrated to be virtually identical to that of the Ras-binding region of c-Raf, in spite of a very low level of sequence identity (13%) (14, 15, 17). Evolutionary pressure is obvious on conservation of structure, and sometimes function, not necessarily of sequence. However, the precise meaning of the presence of a structural module similar to the RA domain in the Grb7/10/14 family and in the band 4.1/JEF domain remains to be defined at the present time. Indeed, three-dimensional structures similar to that of the RA domain and corresponding to the ubiquitin α/β

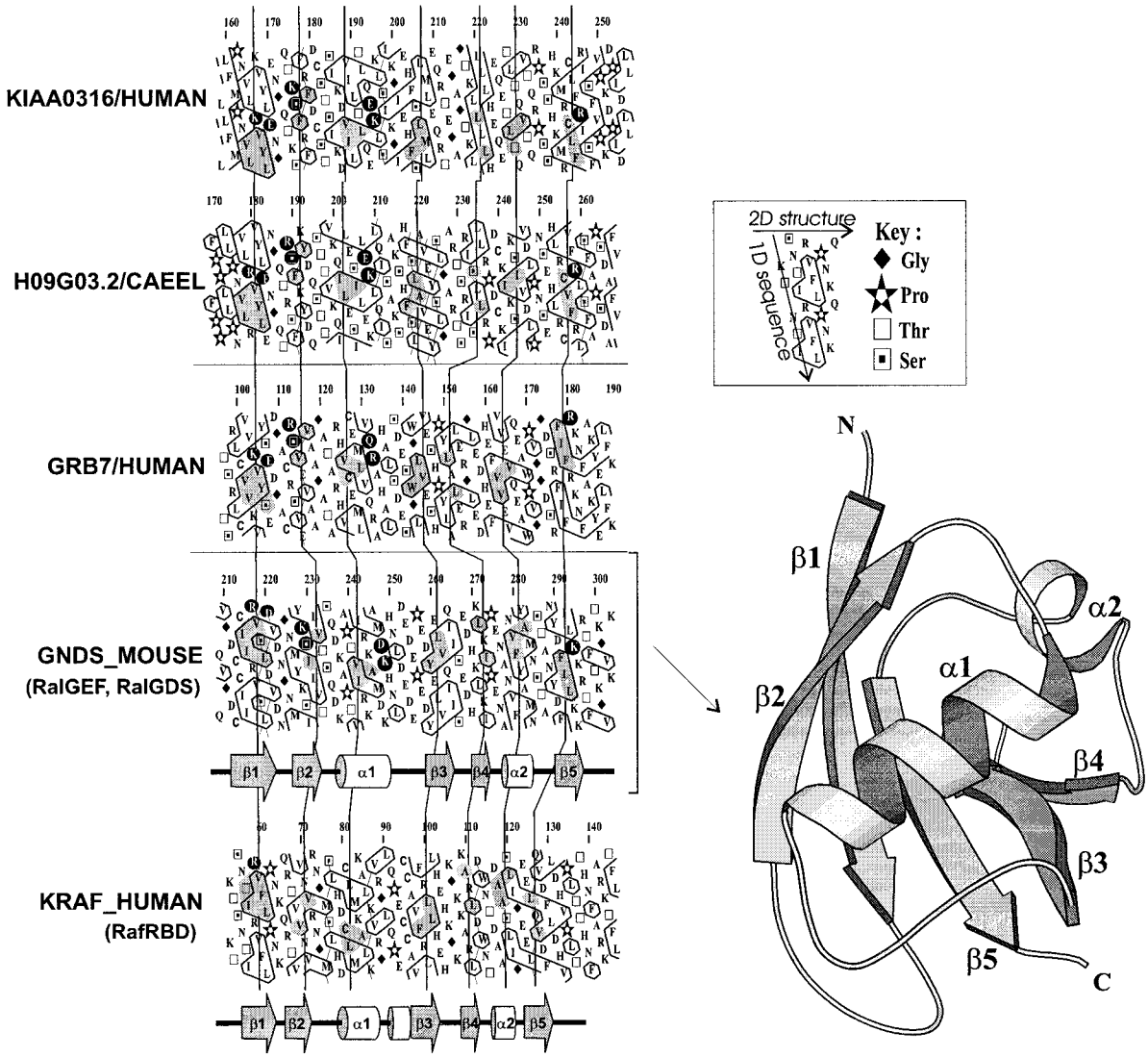

FIG. 2. HCA plot comparison of the RA-like domains of band 4.1/JEF domains from human KIAA0316 and *C. elegans* H09G03.2 as well as of human Grb7 with the Ras-binding domains of mouse RalGDS (GNDS_MOUSE) and human cRaf1 (KRAF_HUMAN), whose three-dimensional structures are known. Briefly, the sequence is shown on a duplicated alpha-helical net. Hydrophobic residues (V, I, L, M, F, Y, W) are boxed and form clusters which mainly correspond to the internal sides of regular secondary structures (65). Special symbols which are used for proline, glycine, serine and threonine are indicated. Sequence identities (white letters on a black background) are associated with hydrophobic cluster similarities (shaded grey), indicating the conservation of a similar local environment. RalGDS and cRaf1 secondary structure positions, as deduced from their experimental structures (PDB identifiers: 1LXD and 1GUA, respectively), are indicated below their HCA plots. The 3D structure of the RA domain of RalGDS is represented on the right, using the MOLSCRIPT software (66).

superfold (18) are found in other proteins which are not functionally related to Ras binding. Hence, the RA-like region of Grb proteins and band 4.1/JEF domains may only correspond to this fold without functional conservation. Such functional versatility has already been observed for other folds involved in signal transduction and interacting with multiple partners (e.g., PH (41, 42) and WW (43) domains). On the other hand it is possible that this RA-like domain has a conserved capacity of interaction with small GTPases. This hypothesis is intriguing since, as discussed below, several proteins with band 4.1/JEF domains and Grb proteins are known to have functional connections with small GTPases.

RalGDS (or RalGEF), one of the best characterised member of the RA family, is one of the Ras effectors, like the protein kinase Raf. These effectors interact much more strongly with the GTP-bound form of the GTPase. The Ras-interacting domains of RalGDS and Raf have a similar structure and their mode of interaction is also strikingly similar. It involves strands of both the effector and the small G protein in an intermolecular β-sheet, with direct participation of the effector strands β1, β2, β5 as well as the effector

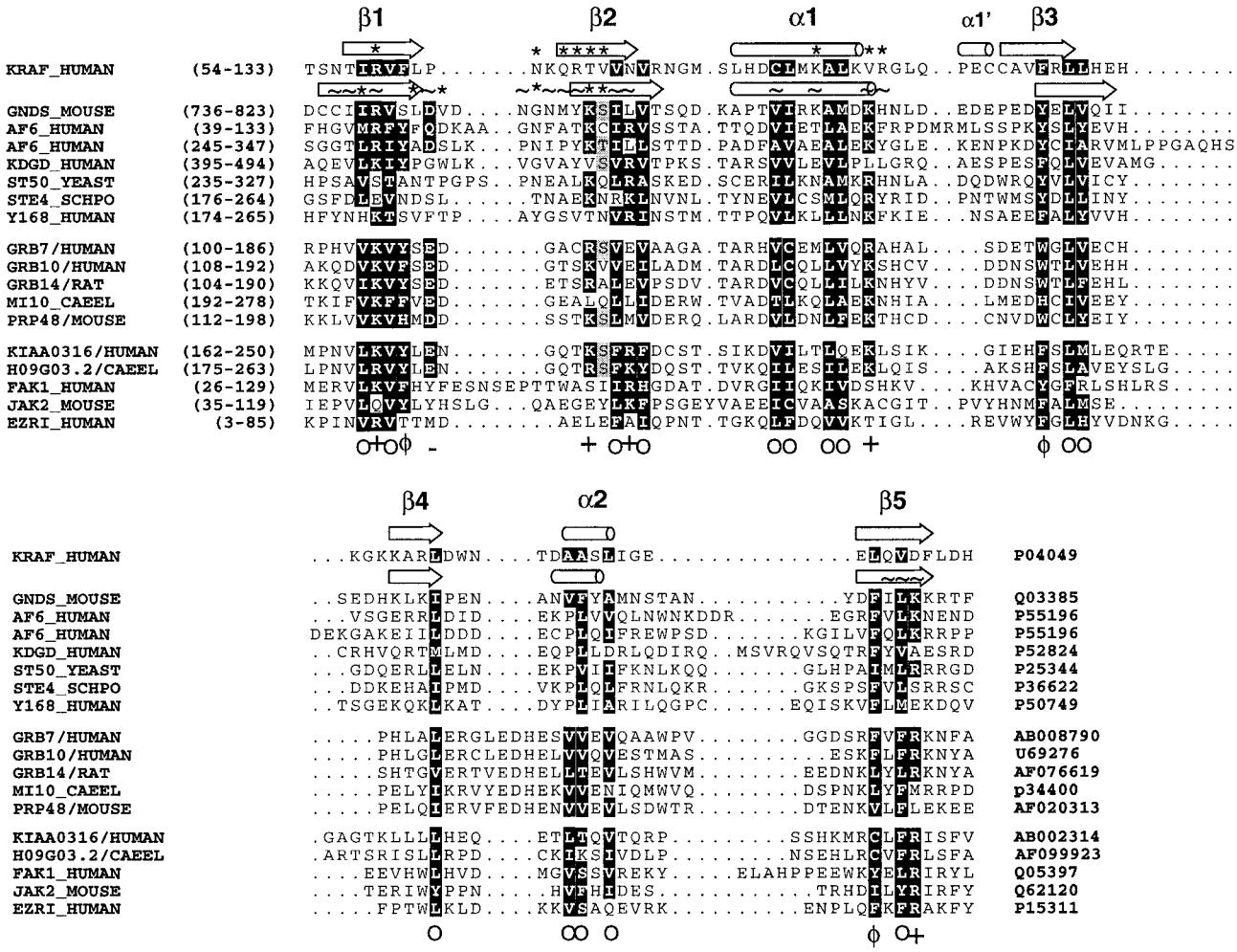

FIG. 3. Multiple alignment of RA domains extracted from the Pfam database (Pfam accession number PF0788) with the RA-like domains of the Grb7/10/14 and band 4.1/JEF families. The structural alignment of the cRaf1 Ras-Binding Domain (RBD, KRAF_HUMAN) with the RalGDS RA domain (GNDS_MOUSE) is also shown (15). The Raf and RalGDS secondary structure positions, as deduced from their experimental structures (PDB identifiers 1GUA and 1LXD, respectively), are indicated at the top of the sequences. Raf and RalGDS residues which are involved in the interaction with Rap1A and Ras, respectively, are indicated by * or ~, according to their degree of participation (15). The consensus line indicates residues which are particularly conserved between the four groups of proteins (O, ϕ, +, - indicate hydrophobic, aromatic, basic and acidic residues, respectively). Representative sequence identities on the aligned segments between the three protein groups are exemplified by the following pairs (GNDS_MOUSE vs GRB7/HUMAN: 15%; GNDS_MOUSE vs PRP48/MOUSE: 20%; GNDS_MOUSE vs JAK2_MOUSE 16%; GRB7/HUMAN vs KIAA0316/HUMAN 25%). GenBank or SwissProt accession numbers are given in the far right column. CAEEL: *Caenorhabditis elegans*, HUMAN: *Homo sapiens*. This figure has been drawn using the ESPript software (67). A comprehensive multiple alignment of RA-like domain sequences of the Grb7/10/14 and JEF/band 4.1 domains is available at http://www.lmcp.jussieu.fr/~callebau/JEF.html.

C-terminal part of the helix α1 (see Figure 2) (14-17). It is worth noting that the sequences corresponding to this set of secondary structure elements in the RA family are those which are the most conserved in the Grb7/10/14 and band 4.1/JEF families, whereas those of strand β4 and helix α2, which are distant from the Ras-binding site, are more divergent (see Figures 2 and 3). Moreover, residues of RalGDS and c-Raf1 involved in complex formation with Ras and Rap1A, respectively (15) (* and ~ on Figure 3), are relatively well conserved in the Grb7/10/14 and band 4.1/JEF families, in particular basic residues. In the RalGDS/Ras (16) and c-Raf1/Rap1A (17) complexes, these residues form a positively charged surface on the Ras-binding domain which is complementary to the small G protein negatively charged surface in the effector region. It should be noted however that despite this striking similarity, which is likely to be the structural basis for interaction between Ras and its two effectors, significant structural differences also exist, probably accounting for the specificity with which Ras distinguishes between them (16). Together, these observations suggest that members of the Grb7/10/14 and band 4.1/JEF families might also bind proteins of

the Ras superfamily through their RA-like domains. Further work is however needed to confirm such a hypothesis.

In the case of the band 4.1/JEF family, the hypothetical presence of a module which might directly bind a member of the Ras superfamily, has interesting implications since several members of this family are regulated by small G proteins, in particular Rho. For instance, binding of ERM proteins (ezrin, radixin, moesin) to transmembrane proteins has been reported to be enhanced by Rho-GTP (44), whereas the effects of active Rho have been shown to require the presence of ERM proteins (45). The exact relation between Rho and ERM proteins is not fully understood. On one hand, Rho kinase, which is activated by Rho, phosphorylates ERM proteins and may maintain them in an active conformation by decreasing head-to-tail interaction (46). On the other hand, the N-ERMAD (N-terminal Ezrin-Radixin-Moesin-Association Domain) of ERM proteins (which correspond to the band 4.1/JEF domain) interacts with RhoGDI and may thus contribute to the activation of the Rho signalling pathway (47). Moreover, radixin interacts with Dbl, a Rho GEF, and it has been suggested that this facilitates the formation of a multimolecular complex consisting of F-actin, radixin, RhoGDI, CD44, Rho-GTP and Rho-target (48). Thus, a direct interaction between Rho-GTP (or a related GTPase) and ERM proteins could participate in the formation of such complexes.

A second group of proteins containing a band 4.1/JEF domain are the FAKs which encompass FAK itself and the related PYK2/CAKβ (see 49 and 50 for recent reviews). FAK is translocated to focal adhesions and autophosphorylated in response to integrin engagement, and appears to play an important role in the antiapoptotic effects of integrins as well as in the control of cell motility. FAK is also regulated in response to stimulation of a number of heterotrimeric G protein-coupled receptors. In many instances, activation of FAK by these receptors has been shown to require Rho (51). Thus, FAK appears also to be downstream of Rho, although the precise molecular mechanism coupling Rho to FAK is unknown and may be indirect. Our observations suggest that FAK may be directly interacting with Rho-GTP, or a related GTPase, through the RA-like region located N-terminus within its band 4.1/JEF domain. As in the case of ERM proteins, this possibility deserves experimental testing.

If we admit that the N-terminal third of band 4.1/JEF domains consists of a RA-like domain, as strongly suggested by the present results, it is remarkable that the band 4.1/JEF domain has been conserved as a whole in a large variety of proteins of many eukaryotic organisms (19, 20). This indicates that in these proteins, the RA-like domain does not function independently, but, rather, in combination with the two other thirds of the band 4.1/JEF domain. Thus, it is possible that the RA-like domain of the band 4.1/JEF domain has a divergent, specialised function, different from that of "isolated" RA domains. In this respect, it is interesting to note that the region of the JAKs which would correspond to the RA-like domain (JH7) is critical for the specificity of the binding of the tyrosine kinase to cytokine receptors (52-57). In particular, a naturally occurring JAK3 mutation (Y100C in JH7) from a patient with autosomal severe combined immunodeficiency (SCID) prevents kinase-receptor association (58). This mutation, located at a position always occupied by a hydrophobic residue in the highly conserved block 6 of the band 4.1/JEF domain (19), should be located in the strand β5 of the RA-like domain, at a position also always occupied by a hydrophobic residue (Y114 in the JAK2_MOUSE sequence of the Figure 3) participating in the proper folding of the domain. To our knowledge, no experimental evidence suggests a role for small GTPases in the kinase-receptor interaction.

The Grb7/10 /14 proteins are currently classified as adapter proteins, which have been isolated on the basis of the ability of their C-terminal SH2 domains to recognise phosphotyrosine sequences in a wide variety of activated tyrosine kinase receptors (27, 59). Interestingly, Grb10 can also interact with receptors devoid of intrinsic tyrosine kinase activity, as recently shown in the case of growth hormone receptor (60). In addition, the Grb7/10/14 proteins contain a small proline-rich region in the N-terminus part which provides a binding site for SH3 domains (30), and a Pleckstrin Homology (PH) domain, located in the central conserved GM region, which might be involved in protein-protein or protein-lipid interaction (see Figure 1). Another region, located Between the PH and SH2 domains (BPS), also named IPS (Insert between the PH and SH2 domains) (61), has been recently shown to interact with insulin and insulin-like growth factor receptors (61, 62). The identification of RA-like domains in the Grb7/10/14 family, which might therefore bind Ras or members of the Ras superfamily, could also shed a new light into the function of these proteins. Indeed, the receptors which are recognised by this family are known to activate the mitogenic MAP kinase signal transduction pathway (63). Interestingly, it has been shown that the SH2 domain of Grb10 interacts with two members of this pathway, namely Raf1 and MEK1, in a phosphotyrosine-independent manner (64). The presence of a RA-like domain leads us to hypothesise that these proteins might be more than adapters or scaffolding proteins and may act as effectors of Ras-GTP.

Thus, the finding of a RA-like domain in two families of proteins which are important for cell structure and signal transduction leads to interesting hypothesis concerning the possible role of small GTPases in the function of these proteins. However, these observations are based on sequence analysis and fold recognition,

and further biochemical experimental work is required to determine whether these RA-like regions have conserved their ability to bind Ras superfamily proteins, or whether their conservation reflects only structural constraints.

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