Current Biology, Vol. 12, 1336–1341, August 6, 2002, ©2002 Elsevier Science Ltd. All rights reserved. PII S0960-9822(02)01038-2

A High-Affinity Arg-X-X-Lys SH3 Binding Motif Confers Specificity for the Interaction between Gads and SLP-76 in T Cell Signaling

Donna M. Berry,[1,2,6] Piers Nash,[3,4,6] Stanley K.-W. Liu,[1,2] Tony Pawson,[3,4] and C. Jane McGlade[1,2,5]

[1]The Arthur and Sonia Labatt
Brain Tumour Research Centre
The Hospital for Sick Children
Toronto, Ontario M5G1X8
Canada

[2]Department of Medical Biophysics

[3]Department of Medical Genetics and Microbiology
University of Toronto
Toronto, Ontario M5S 1A8
Canada

[4]Programme in Molecular Biology and Cancer
Samuel Lunenfeld Research Institute
Mount Sinai Hospital
Toronto, Ontario M5G 1X5
Canada

Summary

A critical event in T cell receptor (TCR)-mediated signaling is the recruitment of hematopoietic-specific adaptor proteins that collect and transmit signals downstream of the TCR [1–3]. Gads, a member of the Grb2 family of SH2 and SH3 domain-containing adaptors, mediates the formation of a complex between LAT and SLP-76 that is essential for signal propagation from the TCR [4–7]. Here we examine the binding specificity of the Gads and Grb2 SH3 domains using peptide arrays and find that a nonproline-based R-X-X-K motif found in SLP-76 binds to the Gads carboxy-terminal SH3 domain with high affinity ($K_D = 240 \pm 45$ nM). The Grb2 C-terminal SH3 domain also binds this motif, but with a 40-fold lower affinity than Gads. Single point mutations in either the relevant R (237) or K (240) completely abrogated SLP-76 association with Gads in vivo and impaired SLP-76 function. A chimeric Grb2 protein, possessing the C-terminal SH3 domain of Gads, was able to partially substitute for Gads in signaling downstream of the T cell receptor. These results provide a molecular explanation for the specific role of Gads in T cell receptor signaling, and identify a discrete subclass of SH3 domains whose binding is dependent on a core R-X-X-K motif.

Results and Discussion

The Gads SH3 Domain Binds an R-X-X-K Amino Acid Motif Found in SLP-76 and Other Gads Binding Proteins

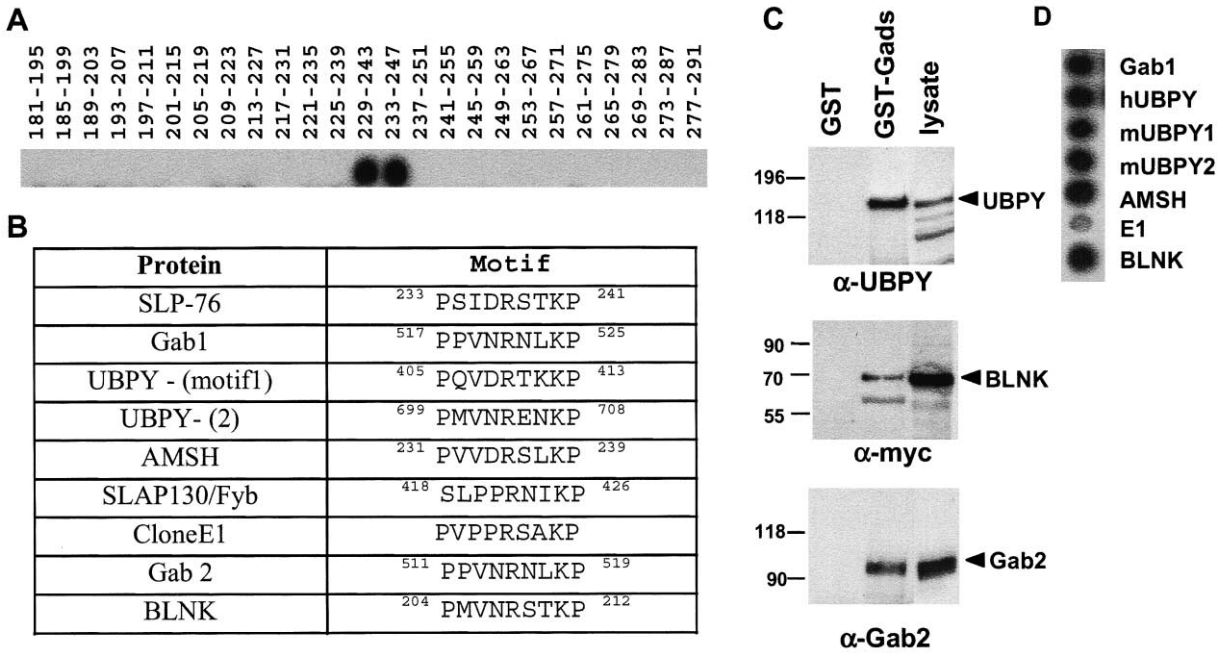
In T cells, SLP-76 is the major binding partner of the Gads C-SH3 domain [4–7]. Previously, we mapped the Gads binding site to a region of SLP-76 (between amino acids 224 and 244) that does not contain a canonical P-X-X-P SH3 domain binding consensus motif [4]. The same region was previously shown to mediate the in vitro binding of Grb2 to SLP-76 [8]. In order to define the motif responsible for the selective recognition of SLP–76 by Gads, a series of overlapping peptides with a 4 amino acid interval were synthesized in solid phase on cellulose filters [9]. The membrane was then incubated with purified recombinant GST-Gads protein. The Gads fusion protein bound specifically to peptides containing amino acids (aa) 233–241, delineating the region that is necessary and sufficient to bind Gads in vitro (Figure 1A). The isolated C-SH3 domain of Gads also bound to the same two peptides, while the amino-terminal SH3 domain did not bind appreciably to any of the SLP-76 peptides tested (data not shown).

This region of SLP-76 does not conform to the canonical SH3 binding motif P-X-X-P, but rather is similar to the atypical Grb2 SH3 binding region of Gab1 [10, 11] and bears substantial similarity to a sequence motif found in Gads binding proteins identified when ^{32}P-labeled GST-Gads was used to screen a 16-day mouse embryo expression library (Figure 1B and see the Supplementary Material available with this article online). These results are consistent with previous studies showing that Gads interacts with Gab1 and AMSH in vitro [6, 10, 12]. The novel interactions between Gads and UBPY, BLNK, and Gab2 were confirmed by in vitro binding experiments using GST-Gads fusion proteins. GST-Gads, but not GST alone, bound to UBPY, Gab2, and BLNK (Figure 1C). To verify that the peptide motifs in these proteins could support Gads binding, peptides corresponding to the binding motifs depicted in Figure 1B were synthesized on filters [13] and probed with purified GST-Gads-C-SH3. All of the peptides tested, including both motifs in UBPY aa 405–413 or 699–708, BLNK aa 204–212, AMSH aa 231–239, and Gab1 aa 517–525, bound to the Gads C-SH3 domain (Figure 1D). Thus, the C-SH3 domain of Gads binds to an atypical SH3 domain binding motif in SLP-76 that is also found in additional signaling proteins.

Based on the optimal binding region in SLP-76, a series of peptides was generated containing an alanine substitution at each position from alanine 232 to isoleucine 242. Only arginine 237 and lysine 240 residues were necessary for GST-Gads binding (Figure 2A). Alanine substitution of any of the other residues, including the conserved proline residues, had no obvious effect on Gads binding, implying that the core binding motif for the Gads C-SH3 domain is R-X-X-K.

To assess the importance of these residues for Gads binding to SLP-76 in vivo, mutant forms of SLP-76 containing alanine substitutions of residues P233, R237, K240, and P241 were generated. Wild-type and mutant forms of SLP-76 were expressed in Jurkat T cells and tested for their ability to bind to endogenous Gads. Mutation of either the arginine (R237A) or lysine (K240A) completely abrogated coimmunoprecipitation of SLP-76 with Gads in transfected cells, while mutation of either proline residue significantly reduced binding (Figure 2B).

[5]Correspondence: jmcglade@sickkids.on.ca
[6]These authors contributed equally to this work.



Protein	Motif
SLP-76	233 PSIDRSTKP 241
Gab1	517 PPVNRNLKP 525
UBPY - (motif1)	405 PQVDRTKKP 413
UBPY- (2)	699 PMVNRENKP 708
AMSH	231 PVVDRSLKP 239
SLAP130/Fyb	418 SLPPRNIKP 426
CloneE1	PVPPRSAKP
Gab 2	511 PPVNRNLKP 519
BLNK	204 PMVNRSTKP 212

Figure 1. Gads Binding Proteins Share an Amino Acid Motif Similar to SLP-76

(A) Refinement of the minimal Gads SH3 domain binding motif in SLP-76. Peptides (15-mer), scanning through the binding region of SLP-76, were synthesized and spotted on a cellulose filter as previously described [9]. The filter was blocked with 5% skim milk powder in TBS-T and probed with full-length GST-Gads C-SH3 fusion protein (2 μg/ml for 3 hr). Bound fusion protein was detected by blotting with monoclonal anti-GST antibody.

(B) Alignment of known and novel Gads SH3 domain binding proteins reveals a common binding motif. A 16-day mouse embryo protein library (Novagen) was screened with GST-Gads fusion protein (see Supplementary Material). Isolated cDNAs were partially sequenced and identified through the GenBank database. A search of the GenBank database for other proteins with the consensus PxxxR-X-X-KP identified both Gab2 and BLNK.

(C) UBPY, Gab2, and BLNK can associate with Gads. Cos1 cells were transfected with 2 μg of pCMV-UBPY or pPuro-BLNK-Myc and harvested 24–48 hr later. Clarified cell lysate from transfected Cos1 cells or from K562 cells was incubated with 2 μg of GST or GST-Gads bound to glutathione-sepharose beads for 1–2 hr at 4°C. Beads were washed with NP-40 lysis buffer, and bound proteins were separated by SDS-PAGE and Western blotted with anti-UBPY (a gift from Paolo Di Fiore and Guillio Draetta), anti-myc (to detect myc-BLNK), or anti-Gab2 (Upstate Biotechnology).

(D) Confirmation of binding sites in UBPY, AMSH, and BLNK. Peptides representing the predicted binding region of each protein were synthesized and spotted onto a cellulose filter as in (A). The filters were probed with Gads GST-C-SH3 fusion protein (2 μg/ml) and blotted with anti-GST monoclonal antibody (Upstate Biotechnology).

These results show that the R-X-X-K motif in SLP-76 is essential for Gads C-SH3 binding in vivo. Additionally, we determined that SLP-76 mutants P233A, R237A, K240A, and P241A fail to complex with phosphorylated LAT following TCR stimulation, correlating with diminished Gads binding (data not shown).

While the flanking prolines are not absolutely required for binding, their presence appears to significantly enhance the Gads-SLP-76 interaction. To determine whether the proline residues form part of an extended peptide binding site, we synthesized SLP-76 peptides in which each of the critical residues was replaced by alanine, and we tested the ability of the mutant peptides to compete with the wild-type sequence for binding to Gads C-SH3 (Figure 2C). Peptides with alanine substitutions of either R237 or K240 failed to measurably compete for binding, while peptides with substitutions of either proline residue P233 or P241 were able to compete with the wild-type sequence, albeit with reduced efficiency (IC_{50} = 2.4 and 4.3 μM, respectively). Substitution of both proline residues significantly impaired the binding of this peptide to Gads C-SH3 (IC_{50} = 230 μM). These results confirm that, while the proline residues are not strictly essential for Gads C-SH3 domain recognition, they contribute to the affinity of the interaction.

Transcription of the IL-2 gene following TCR ligation is an essential component of the T cell activation process. This event is mediated by binding of the activated NFAT transcription factor to the IL-2 promoter. In Jurkat T cells, SLP-76 is required for activation of a luciferase reporter gene driven by the NFAT binding region of the IL-2 promoter in response to TCR ligation [8]. To examine the functional role of the R-X-X-K motif in SLP-76, Jurkat T cells deficient in SLP-76 (clone J14), were transiently cotransfected with wild-type or mutant SLP-76 and an NFAT-luciferase reporter gene (Figure 2D). Compared to wild-type, SLP-76 mutants that did not bind to Gads (R237A and K240A) were severely compromised in their ability to promote NFAT activation, while SLP-76 with mutations of the proline residues 233 or 241 rescued NFAT activation, but to a lesser degree than wild-type SLP-76. Although the R237A and K240A mutants are defective in their ability to activate NFAT, they still retain detectable activity in this assay. This residual

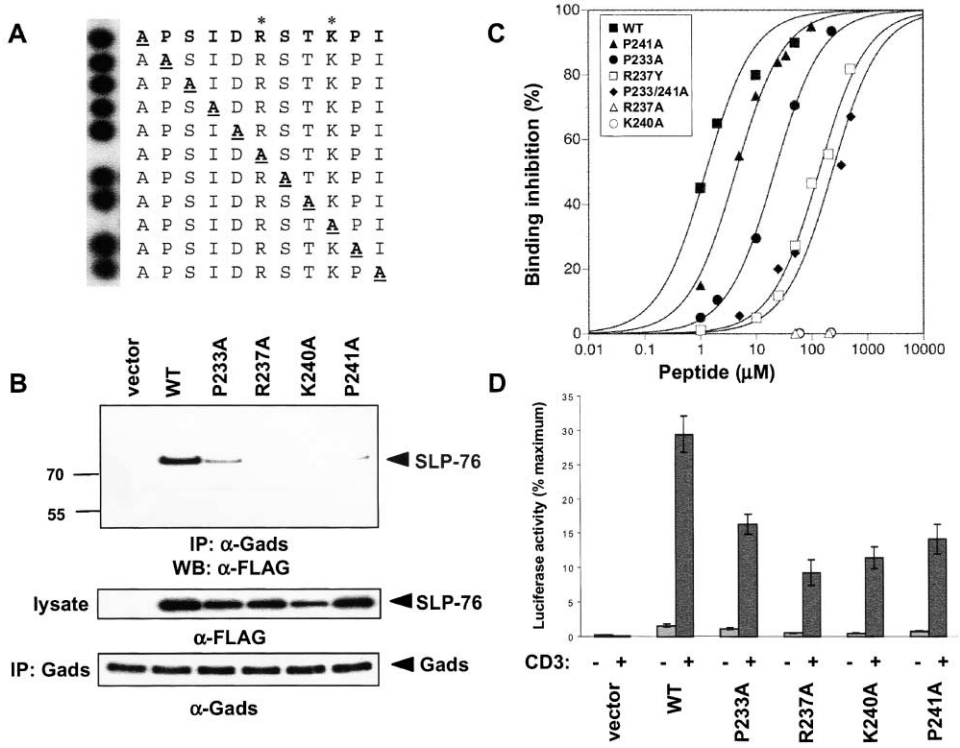


Figure 2. Assignment of an Atypical Binding Motif for the Gads SH3 Domain

(A) SLP-76 peptides were synthesized and spotted on a cellulose filter as described [9] with alanine substitutions for each amino acid in the binding motif ^{233}PSIDRSTKP^{241}. The filter was probed with full-length GST-Gads C-SH3 fusion protein as described in Figure 1A.

(B) SLP-76 constructs containing alanine substitutions at P233, R237, K240, and P241 were generated by PCR and cloned into pEF-Flag. Jurkat T cells were electroporated with 40 μg of pEF-wild-type or mutant SLP-76, as described previously [4], and anti-Gads immunoprecipitations were performed using clarified lysates. Coimmunoprecipitating SLP-76 was detected by Western blot using a monoclonal anti-Flag antibody (Sigma) (top). Equal expression of SLP-76 mutants was assessed by anti-FLAG Western blotting of total cell lysates (middle), and the Gads immunoprecipitates were reprobed with polyclonal anti-Gads antibody to show equal precipitation of Gads (panel).

(C) Peptide IC_{50} curves for the competition of binding to Gads C-SH3. Fluorescein-SLP-76wt peptide (Fl-APSIDRSTKPA) bound to 300 nM Gads C-SH3 was competed away by SLP-76wt (■), SLP-76 P233A (●), SLP-76 P241A (▲), SLP-76 PP233,241AA (◆), SLP-76 R237Y (□), SLP-76 K240A (○), and SLP-76 R237A (△) as previously described [18]. Calculated IC_{50} values were obtained from the average of at least three independent experiments.

(D) SLP-76-deficient Jurkat cells (clone J14) (2×10^7) were electroporated with 20 μg NFAT luciferase reporter construct, and 40 μg of empty pEF vector or 40 μg of Flag epitope-tagged, SLP-76 wt, or mutants. Twenty-four hours after transfection, 5×10^5 cells (in triplicate) were stimulated with anti-CD3, anti-CD3 + PMA, or Ionomycin + PMA for 8 hr at 37°C, and cell lysates were prepared as previously described [4]. For a minimum of three separate experiments, luciferase activity was quantified with a luminometer as previously described [4] and expressed as a percentage of maximum stimulation. Error bars indicate standard error of the mean (SEM).

activity is indicative of the known capacity of SLP-76 to recruit additional signaling molecules such as SLAP-130(ADAP), Nck, and Vav that couple the activated TCR to distinct downstream effector pathways [14]. Furthermore, SLP-76 has been shown to associate with PLCγ directly, and therefore it could retain some ability to induce NFAT activation even in the complete absence of Gads association [15].

Both Gads and Grb2 Carboxy-Terminal SH3 Domains Bind R-X-X-K Motifs

The region of SLP-76 that contains the R-X-X-K motif has also been demonstrated to contain the in vitro binding site for the Grb2 C-SH3 domain. To further define the distinct binding characteristics of Gads and Grb2 SH3 domains, a peptide spots array consisting of 180 individual peptides was employed. The peptide corresponding to the Gads binding site on SLP-76 was used as a template in which each residue in the sequence was substituted with each of the 20 natural amino acids. The immobilized peptide arrays were then probed with either GST-Gads-C-SH3 or GST-Grb2-C-SH3 fusion proteins (Figures 3A and 3B). Binding of the Gads SH3 domain allowed virtually no substitutions of the R237 or K240 residue and only noncharged residues at the −2 (235) position. The proline residues at positions 233 and 241 were dispensable, and binding to the Gads C-SH3 was retained when they were exchanged for any amino acid (Figure 3A). These results are supported by IC_{50} measurements with selected mutant peptides (Figure 2C). These data confirm the essential nature of R237 and K240 and the more minor role of P233 and P241. The substitution of a tyrosine residue in place of R237 that supports marginal binding on the peptide array spots blot supports only low-affinity binding in solution, affording an IC_{50} of 135 μM (Figure 2C). Similar to Gads, the Grb2-C-SH3 domain also absolutely required the conserved R and K residues; however, certain residues

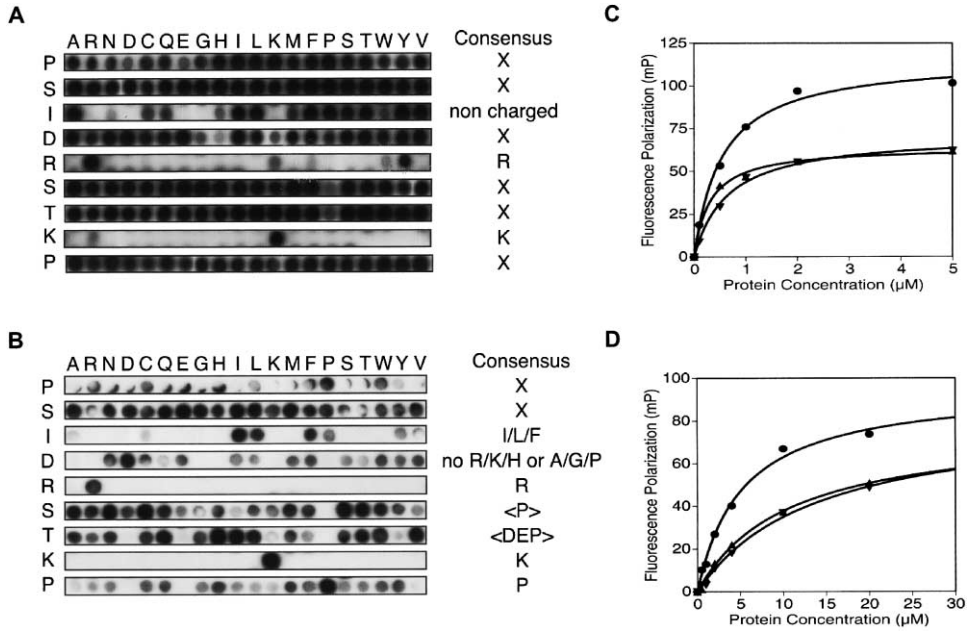


Figure 3. An R-X-X-K Motif Is Essential for Both Gads and Grb-2 Carboxy-Terminal SH3 Domain Recognition

(A and B) Peptide arrays were constructed according to the Spots synthesis method as previously described [9]. Each amino acid of the SLP-76 binding motif (vertical axis) was substituted with each natural amino acid (horizontal axis). Membranes were blocked overnight in 5% skim milk and incubated with (A) 2 μg/ml of GST-Gads-C-SH3 or (B) GST-Grb2-C-SH3 fusion proteins, and bound protein was detected as described for Figure 1.

(C and D) GST-SH3 domain fusion proteins were produced and purified as described previously, and the GST tag was cleaved from the protein using thrombin (Sigma) as per manufacturer's instructions. Purified SH3 domain preparations were incubated several times with benzamidine sepharose (Amersham Pharmacia) beads to remove excess thrombin and yield a pure sample for affinity measurements. Peptides were synthesized as described previously [19]. Purification was accomplished by HPLC and peptide identity confirmed by MALDI mass spectrometry. Equilibrium binding constant determination was carried out using fluorescence polarization as previously described [18]. Michaelis-Menton plot for (C) GST Gads C-SH3 and (D) GST Grb2 C-SH3 interaction with peptides corresponding to amino acids 232–241 of SLP-76 with sequence APSIDRSTKPA (▲); amino acids 203–213 of BLNK with sequence APMVNRSTKPN (▼); and amino acids 516–526 of Gab1 with sequence PPPVDRNLKPD (●). Plots are of a representative experiment. K_D values were calculated from the average of at least three independent experiments and errors are SEM.

were strongly selected at additional positions. For instance, a strong preference for isoleucine, leucine, or phenylalanine existed at position −2, relative to the conserved arginine, suggesting a possible hydrophobic pocket interaction at this position. Interestingly, a strong preference against proline at positions −1, +1, +2, and +4 relative to the conserved arginine is also evident for Grb2. The Gads SH3 domain also accepted any amino acid, except proline, at positions +1 and +2. This is particularly striking as this indicates that the creation of a conventional P-X-X-P ligand within the context of the SLP-76 peptide is not favorable for binding to either Gads or Grb2 SH3 domains. Thus, future structural examination of the binding of this class of SH3 domains to R-X-X-K peptides will be particularly interesting.

Since the C-SH3 domains of both Grb2 and Gads appear to bind to the conserved R-X-X-K motif present in SLP-76, the question remained as to why only a Gads-SLP-76 interaction occurs in vivo. To address this question, the binding affinities of Grb2 and Gads carboxy-terminal SH3 domains for SLP-76, Gab1, and Blnk peptides were assessed. Fluorescein-labeled peptides corresponding to the R-X-X-K-containing regions of SLP-76, Gab1, and BLNK were synthesized and employed as probes to measure the equilibrium dissociation constants for binding to the C-SH3 domains of Gads and Grb2 in solution by fluorescence polarization (Figures 3C and 3D). The Gads C-SH3 domain bound to SLP-76, Gab1, and BLNK peptides with $K_D = 240 \pm 45$ nM, 550 ± 70 nM, and 610 ± 80 nM, respectively (Figure 3C). By contrast, the Grb2 C-SH3 domain bound to SLP-76, Gab1, and BLNK peptides with $K_D = 10.8 \pm 2$ μM, 5.1 ± 0.8 μM, and 13 ± 3 μM, respectively. Thus, the relative affinity of the Gads C-SH3 domain for SLP-76 is approximately 40-fold higher than that of the Grb2 C-SH3 domain for the same peptide. This 40-fold difference in binding affinity suggests that in T cells, where both Grb2 and Gads are expressed, the specificity as well as the relative affinity of the C-SH3 domain of Gads regulates the formation of Gads-SLP-76 complexes. In vitro, the affinity of the Gads C-SH3 domain for two well-known Grb2 binding partners BLNK and Gab1 is also higher than that of the Grb2 SH3 domain, indicating that other factors influence the formation of Grb2 versus Gads complexes with these targets.

The Gads SH3 Domain Confers SLP-76 Binding and TCR Signaling Properties to Grb2

On the basis of the observations above, indicating that the carboxy-terminal SH3 domain of Gads mediates its specific function in TCR signaling, we examined whether substitution of the carboxy-terminal SH3 domain of Grb2

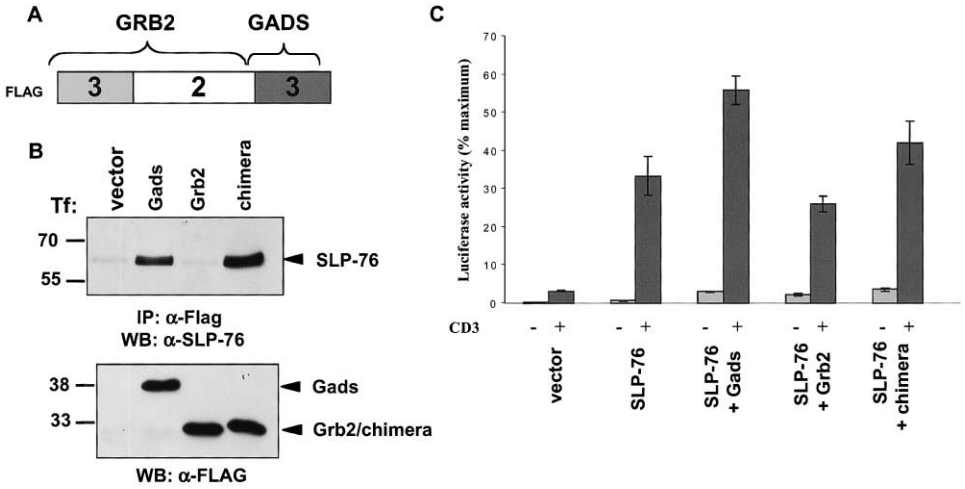


Figure 4. The Gads SH3 Domain Confers SLP-76 Binding and TCR Signaling Properties to Grb2

(A) Schematic of the Grb2-Gads chimera that was generated by PCR amplification and ligation of cDNA fragments encoding amino acids 1–154 of Grb2 (N-SH3 and SH2) and amino acids 257–322 of Gads (C-SH3). The chimeric cDNA was cloned into a pEF vector with a Flag epitope tag.

(B) Jurkat T cells were electroporated with 40 μg of either empty pEF vector, or pEF containing full-length Gads, Grb2, or the chimera. Anti-Flag immunoprecipitations were performed on clarified lysates, and Western blots were probed with polyclonal anti-SLP-76 anti-sera (a gift from Gary Koretzky) (top). Total cell lysates from each condition were Western blotted with anti-Flag antibody (Sigma) to assess expression levels of the transfected proteins.

(C) Grb2-Gads chimera synergizes with SLP-76 to activate NFAT downstream of the TCR. Jurkat cells (2×10^7) were electroporated with 20 μg NFAT-luciferase reporter construct, together with 40 μg of empty pEF vector or 40 μg of Flag epitope-tagged Gads, Grb2, or a Grb2 construct with the C-terminal SH3 of Gads. Luciferase assays were performed and data presented as described for Figure 2C.

with that of Gads would confer Gads specific function by generating a Grb2-Gads(C-SH3) chimera (Figure 4A). Flag epitope-tagged wild-type Grb2, Gads, and the chimeric Grb2-Gads(C-SH3) were transiently expressed in Jurkat cells and immunoprecipitated with anti-FLAG antibodies. SLP-76 did not bind to wild-type Grb2, whereas both Gads and the chimera coimmunoprecipitated endogenous SLP-76 (Figure 4B). To determine whether the chimera is functional in TCR signaling, we expressed the chimera in Jurkat T cells and measured NFAT activation, as a distal indicator of TCR activation. As previously reported, Gads synergized with SLP-76 to enhance NFAT while Grb2 coexpression had no effect (Figure 4C). When the C-SH3 domain of Grb2 was replaced with the C-SH3 of Gads, the resulting chimera was able to synergize with SLP-76, suggesting that the ability of Gads to act downstream of the TCR is due to the high-affinity binding of its C-SH3 domain to SLP-76.

In conclusion, we have shown that Gads function in TCR signaling is defined by the specificity and affinity of its carboxy-terminal SH3 domain for a novel R-X-X-K motif in SLP-76. Despite the similarities in binding specificity between Gads and Grb2, the 40-fold lower affinity of the Grb2 SH3 for SLP-76 renders it unable to carry out a similar function. These results provide a molecular explanation for the specific function of Gads to facilitate T cell receptor signaling by forming a trimolecular complex with SLP-76 and LAT.

Furthermore, our results suggest that the carboxy-terminal SH3 domains of Gads and Grb2 define a family of SH3 domains, which also includes those found in STAM and in Hbp (STAM2), that bind to R-X-X-K motifs rather than the canonical P-X-X-P SH3 domain binding motif [10, 16, 17]. Phylogenetic analysis of the complete set of 211 nonredundant human SH3 domains indicates that Grb2 C-SH3, Gads C-SH3, Grap C-SH3, STAM, and Hbp (STAM2) SH3 domains cluster as a group comprised of 16 SH3 domains that separate from the bulk of the SH3 domains at the first branch point (Supplementary Material). Therefore, we suggest that the carboxy-terminal SH3 domains of Gads, Grb2, and Grap, the STAM and HBP SH3 domains, as well as other SH3 domains with a specificity for cognate peptide ligands with a conserved R-X-X-K motif, may be considered as a separate subclass of SH3 domains.

Supplementary Material

Supplementary material, including methods for expression library screening, and a supplementary figure depicting phylogenetic analysis of SH3 domains is available at http://images.cellpress.com/supmat/supmatin.htm.

Acknowledgments

We thank Gary Koretzky for the SLP-76 and SLAP-130 cDNAs and antiserum, Andy Chan for the BLNK cDNA, Paolo Di Fiore and Guillio Draetta for UBPY reagents, Robert Rottapel for the GST-Grb2-SH3C, Mike Moran for the murine Grb2 cDNA, and Monica Raina for technical assistance. P.N. is the recipient of a CIHR senior research fellowship. T.P is a Distinguished Scientist of the CIHR. C.J.M. is a research scientist of the National Cancer Institute of Canada. This work was supported by grants from the Canadian Institutes of Health Research (CIHR), the National Cancer Institute of Canada (NCIC), the Protein Engineering Network of Centers of Excellence (PENCE), and the Cancer Research Society, Inc.

Received: November 26, 2001
Revised: January 5, 2002

Accepted: June 17, 2002
Published: August 6, 2002

References

1. Rudd, C.E. (1999). Adaptors and molecular scaffolds in immune cell signaling. Cell *96*, 5–8.
2. Myung, P.S., Boerthe, N.J., and Koretzky, G.A. (2000). Adapter proteins in lymphocyte antigen-receptor signaling. Curr. Opin. Immunol. *12*, 256–266.
3. Pawson, T., and Nash, P. (2000). Protein-protein interactions define specificity in signal transduction. Genes Dev. *14*, 1027–1047.
4. Liu, S.K., Fang, N., Koretzky, G.A., and McGlade, C.J. (1999). The hematopoietic-specific adaptor protein gads functions in T-cell signaling via interactions with the SLP-76 and LAT adaptors. Curr. Biol. *9*, 67–75.
5. Law, C.L., Ewings, M.K., Chaudhary, P.M., Solow, S.A., Yun, T.J., Marshall, A.J., Hood, L., and Clark, E.A. (1999). GrpL, a Grb2-related adaptor protein, interacts with SLP-76 to regulate nuclear factor of activated T cell activation. J. Exp. Med. *189*, 1243–1253.
6. Asada, H., Ishii, N., Sasaki, Y., Endo, K., Kasai, H., Tanaka, N., Takeshita, T., Tsuchiya, S., Konno, T., and Sugamura, K. (1999). Grf40, a novel grb2 family member, is involved in T cell signaling through interaction with SLP-76 and LAT. J. Exp. Med. *189*, 1383–1390.
7. Yoder, J., Pham, C., Iizuka, Y.M., Kanagawa, O., Liu, S.K., McGlade, J., and Cheng, A.M. (2001). Requirement for the SLP-76 adaptor GADS in T cell development. Science *291*, 1987–1991.
8. Motto, D.G., Ross, S.E., and Wu, J., Hendricks-Taylor, L.R., and Koretzky, G.A. (1996). Implication of the GRB2-associated phosphoprotein SLP-76 in T cell receptor-mediated interleukin 2 production. J. Exp. Med. *183*, 1937–1943.
9. Frank, R. (1992). Spot-synthesis: an easy technique for positionally addressable, parallel chemical synthesis on a membrane support. Tetrahedron *48*, 9217–9232.
10. Lock, L.S., Royal, I., Naujokas, M.A., and Park, M. (2000). Identification of an atypical Grb2 carboxyl-terminal SH3 domain binding site in Gab docking proteins reveals Grb2-dependent and -independent recruitment of Gab1 to receptor tyrosine kinases. J. Biol. Chem. *275*, 31536–31545.
11. Lewitzky, M., Kardinal, C., Gehring, N.H., Schmidt, E.K., Konkol, B., Eulitz, M., Birchmeier, W., Schaeper, U., and Feller, S.M. (2001). The C-terminal SH3 domain of the adapter protein Grb2 binds with high affinity to sequences in Gab1 and SLP-76 which lack the SH3-typical P-x-x-P core motif. Oncogene *20*, 1052–1062.
12. Qiu, M., Hua, S., Agrawal, M., Li, G., Cai, J., Chan, E., Zhou, H., Luo, Y., and Liu, M. (1998). Molecular cloning and expression of human grap-2, a novel leukocyte-specific SH2- and SH3-containing adaptor-like protein that binds to gab-*1*. Biochem. Biophys. Res. Commun. *253*, 443–447.
13. Frank, R., and Overwin, H. (1996). SPOT synthesis. Epitope analysis with arrays of synthetic peptides prepared on cellulose membranes. Methods Mol. Biol. *66*, 149–169.
14. Myung, P.S., Derimanov, G.S., Jordan, M.S., Punt, J.A., Liu, Q.H., Judd, B.A., Meyers, E.E., Sigmund, C.D., Freedman, B.D., and Koretzky, G.A. (2001). Differential requirement for SLP-76 domains in T cell development and function. Immunity *15*, 1011–1026.
15. Yablonski, D., Kadlecek, T., and Weiss, A. (2001). Identification of a phospholipase C-gamma1 (PLC-gamma1) SH3 domain-binding site in SLP-76 required for T-cell receptor-mediated activation of PLC-gamma1 and NFAT. Mol. Cell. Biol. *21*, 4208–4218.
16. Schaeper, U., Gehring, N.H., Fuchs, K.P., Sachs, M., Kempkes, B., and Birchmeier, W. (2000). Coupling of Gab1 to c-Met, Grb2, and Shp2 mediates biological responses. J. Cell Biol. *149*, 1419–1432.
17. Kato, M., Miyazawa, K., and Kitamura, N. (2000). A deubiquitinating enzyme UBPY interacts with the Src homology 3 domain of Hrs-binding protein via a novel binding motif PX(V/I)(D/N)RXXKP. J. Biol. Chem. *275*, 37481–37487.
18. Nash, P., Tang, X., Orlicky, S., Chen, Q., Gertler, F.B., Mendenhall, M.D., Sicheri, F., Pawson, T., and Tyers, M. (2001). Multisite phosphorylation of a CDK inhibitor sets a threshold for the onset of DNA replication. Nature *414*, 514–521.
19. van der Geer, P., Wiley, S., Gish, G.D., and Pawson, T. (1996). The Shc adaptor protein is highly phosphorylated at conserved, twin tyrosine residues (Y239/240) that mediate protein-protein interactions. Curr. Biol. *6*, 1435–1444.