Brief Definitive Report

p95^{vav} Associates with Tyrosine-phosphorylated SLP-76 in Antigen-stimulated T Cells

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Summary

p95^{vav}, the product of the *vav* protooncogene, has been implicated in the T cell receptor (TCR)-mediated signaling cascade. p95^{vav} is phosphorylated on tyrosine residues after TCR stimulation by anti-TCR/CD3 antibodies and possesses a number of landmark features of signaling molecules such as a putative guanine nucleotide exchange factor domain, a pleckstrin homology domain, and an Src homology (SH) 2 and two SH3 domains, which provide the capacity to form multimeric signaling complexes. However, the precise role of p95^{vav} in TCR signaling remains unclear. In this work we show that physiological stimulation of T cell hybridomas with antigen presented by major histocompatibility complex class II molecules leads to a strong tyrosine phosphorylation of p95^{vav} and its association with tyrosine-phosphorylated SLP-76. SLP-76 is a newly described SH2-containing protein that has been previously found to bind to the adapter molecule Grb2. Moreover, we provide evidence that p95^{vav}–SLP-76 association is SH2-mediated by demonstrating that this interaction can be inhibited by a phosphopeptide containing a putative p95^{vav}–SH2–binding motif (pYESP) present in SLP-76. Furthermore, in vitro experiments show that after antigen stimulation, phosphorylated p95^{vav}–SLP-76 can bind to Grb2 in a complex that contains pp36/38 and pp116 proteins. Our data provide a clue to explain recent independent observations that overexpression of p95^{vav} or SLP-76 enhances TCR-mediated gene activation.

Activation of Src and Syk family protein tyrosine kinases (PTKs) with subsequent phosphorylation of a number of cellular substrates ranks among the earliest events coupling the TCR to downstream signaling pathways such as the Ca^{2+}/calcineurin and Ras/Raf-1/mitogen-activated protein (MAP) kinases (1, 2). Although several proteins phosphorylated on tyrosine after TCR triggering have been identified (for review see reference 3), in most cases their relative position in the signaling cascades remains elusive. One of these proteins is p95^{vav} (4, 5), the product of the *vav* protooncogene expressed almost exclusively in cells of hematopoietic origin (6). Although p95^{vav} function in receptor-mediated signaling is not yet fully understood, data from *vav*-deficient mice indicate that it has a role in the control of T and B cell development and activation (7–9). Moreover, the participation of p95^{vav} as a positive regulator of TCR-mediated signaling pathways has been recently suggested by the observation that its overexpression in Jurkat cells led to spontaneous activation of nuclear factor of activated T cells (NFAT) and enhanced TCR-stimulated NFAT activity in a PTK-dependent manner (10, 11).

p95^{vav} contains multiple structural motifs found in intracellular signaling molecules, which include a CDC24/Dbl homology region characteristic of guanine nucleotide exchange factors (GEF) as well as a pleckstrin homology domain, a cystein-rich domain, and Src homology (SH) 2 and SH3 domains that mediate interactions among proteins and the formation of multimeric signaling complexes (6). p95^{vav} was reported to display in vitro guanine nucleotide exchange activity for p21^{ras} after T and B cell stimulation (12). However, recent reports suggest that p95^{vav} acts as a GEF for an unidentified member of the Rho/Rac family rather than for p21^{ras} (13, 14). Moreover, evidence indicates that p95^{vav} interaction with other TCR-induced phosphotyrosine proteins could be implicated in the regulation of its activity (15, 16). In particular, p95^{vav} has been described to interact, via its SH2 domain, with the PTK ZAP-70 in TCR-stimulated Jurkat cells, possibly implicating ZAP-70 in the phosphorylation of p95^{vav} (15). However, other unidentified phosphotyrosine proteins of 80 and 74 kD were seen to coprecipitate after TCR ligation, suggesting that they could be involved in the regulation of p95^{vav} functions.

These studies were carried out in T cell lines stimulated by anti-TCR/CD3 antibodies. We were interested in identifying tyrosine-phosphorylated proteins that associate with p95^{vav} after physiological TCR recognition of Ag–MHC complexes. To this end, we used murine T cell hybridomas specific for a tetanus toxin peptide (17). We found that stimulation by antigen presented by APC induced a strong tyrosine phosphorylation of p95^{vav} and its association with tyrosine-phosphorylated SLP-76, a novel SH2 domain–

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containing protein that was previously described as being associated with Grb2 in activated T cells (18–20). Additionally, we provide evidence that $p95^{vav}$–SLP-76 interaction in SH2 mediated. These results, together with recent reports that overexpression of $p95^{vav}$ or SLP-76 independently increased TCR-mediated induction of NFAT and IL-2 promoter activity (10, 21), suggest a functional role of this interaction.

Materials and Methods

Antibodies, Glutathione-S-Transferase (GST) Fusion Protein, and Peptides. Rabbit polyclonal Ab anti-$p95^{vav}$ and antiphosphotyrosine mAb 4G10 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). The sheep polyclonal Ab anti-SLP76 raised against a portion (residues 136–235) of SLP-76 as a GST fusion protein was kindly provided by Dr. G.A. Koretzky (University of Iowa College of Medicine, Iowa City, IA). The hamster mAb 145-2C11 specific for mouse CD3ε chain was used purified. The following secondary Abs were used in immunoblotting: peroxidase conjugated goat anti–mouse (Amersham International, Amersham, Bucks, UK), peroxidase-conjugated goat anti–rabbit (Biosys, Compiegne, France), and peroxidase-conjugated donkey anti–sheep Abs (Sigma Chemical Co., St. Louis, MO). The GST–Grb2 fusion protein coupled to agarose beads was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The tetanus toxin peptide tt830-843 (QYIKANSKFIGITE) and the peptide TSVYESPYS corresponding to residues 312–320 of human ZAP-70 (>95% pure) were purchased from Neosystem SA (Strasbourg, France). The tyrosine phosphorylated peptide TSVY (PO_4)ESPYS was purchased from Chiron Mimotopes Peptide Systems (Victoria, Australia).

Cell Lines. The murine T cell hybridomas T.AL15.3 and T.AL8.1 expressing human CD4 and anti–tt830-843 TCRs restricted to HLA-DRB1*1102 were previously described (17). The L cell transfectants (L625.7) expressing HLA-DRB1*1102 (22) were kindly provided by Dr. R.W. Karr (Monsanto Chemical Co., St. Louis, MO).

Cell Stimulations, Immunoprecipitations, and Reimmunoprecipitations. For Ag stimulation, T cell hybridomas (10^8/ml) were incubated in suspension for 5 min at 37°C with L625.7 fibroblasts (10^8/ml) prepulsed for 3 h, with the concentration of antigenic peptide tt830-843 (20 μg/ml) inducing maximal production of IL-2 (17). For anti-CD3 stimulation, cells were incubated at 4°C for 30 min with 145-2C11 mAb (10 μg/ml), washed, and incubated at 37°C for 5 min with secondary goat anti–hamster Ab (25 μg/ml; Southern Biotechnology Associates, Birmingham, AL). At the end of incubation, cells were harvested and lysed at 10^8 cells/ml for 30 min on ice in 1% NP-40 lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, and 1 mM EGTA in the presence of inhibitors of proteases and phosphatases: 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM Pefabloc-sc, 50 mM NaF, 10 mM $Na_4P_2O_7$, and 1 mM NaVO4. For peptide competition experiments, unphosphorylated or tyrosine-phosphorylated peptide TSVY(PO_4)ESPYS was added to the lysis buffer at concentrations of 50 and 500 μM. Postnuclear lysates were obtained after centrifugation at 14,000 rpm for 10 min at 4°C and were precleared for 1 h with protein A– or protein G–Sepharose (Pharmacia, Uppsala, Sweden). Precleared lysates were incubated for 1 h with Abs preadsorbed to protein A– or protein G–Sepharose beads. The reimmunoprecipation experiments were performed as previously described (23). Immunoprecipitates were washed twice in 1% detergent lysis buffer and twice in 0.05% detergent lysis buffer and boiled in SDS sample buffer before gel electrophoresis. Immunoblotting and detection of proteins were performed as described (23).

Results and Discussion

TCR Triggering by Ag–MHC Complexes Induces $p95^{vav}$ Tyrosine Phosphorylation and Association with a pp75/76 Phosphoprotein. To analyze phosphotyrosine modifications of T cell intracellular proteins induced by TCR stimulation with Ag–MHC complexes, we used murine T cell hybridomas specific for tt830-843 peptide presented by HLA-DRB1*1102 molecules expressed on L625.7 L cells (17). T.AL15.3 and T.AL8.1 T cell hybridomas were left unstimulated or added for 5 min to L625.7 cells or L625.7 cells prepulsed for 3 h with an optimal concentration (20 μg/ml) of tt830-843 peptide. This peptide concentration induced maximal production of IL-2 in both hybridomas, although higher levels were found for T.AL8.1 (17). At the end of the incubation, cells were lysed in 1% NP-40, and phosphoproteins were analyzed on SDS-PAGE, followed by immunoblotting with antiphosphotyrosine mAbs. A 3:1 ratio of T cell hybridomas to L625.7 cells was used in these experiments to keep the background due to L625.7 cells to an acceptable level (data not shown). As shown in Fig. 1, contact of T cell hybridomas with L625.7 cells alone (*APC*) induced tyrosine phosphorylation of 95- (*pp95*), 116- (*pp116*), and 130-kD (*pp130*) proteins, which increased when the same cells were stimulated in the presence of the Ag (*APC/Ag*). Furthermore, Ag stimulation induced tyrosine phosphorylation of a 75/76-kD protein (*pp75/76*), which migrated as a doublet, of a 70-kD protein (*pp70*) likely to represent ZAP-70, as evidenced by

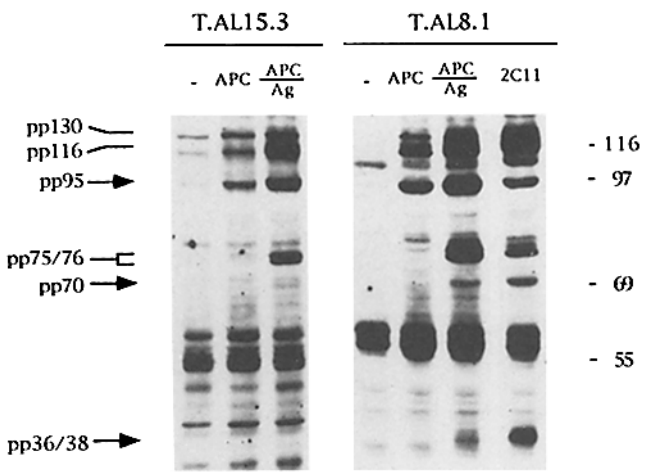

Figure 1. Tyrosine phosphorylation pattern of T cell hybridomas stimulated by Ag–MHC. NP-40 lysates from T.AL15.3 and T.AL8.1 T cell hybridomas unstimulated (−) or activated for 5 min at 37°C with L625.7 cells (*APC*) or L625.7 cells prepulsed with the tt830-843 peptide (*APC/Ag*) or with anti-CD3 Ab (*2C11*) were loaded on an 8% SDS–polyacrylamide gel and analyzed by antiphosphotyrosine immunoblotting. Tyrosine-phosphorylated proteins with their relative molecular mass (in kilodaltons) are indicated on the left. Position of molecular mass markers are shown in kilodaltons.

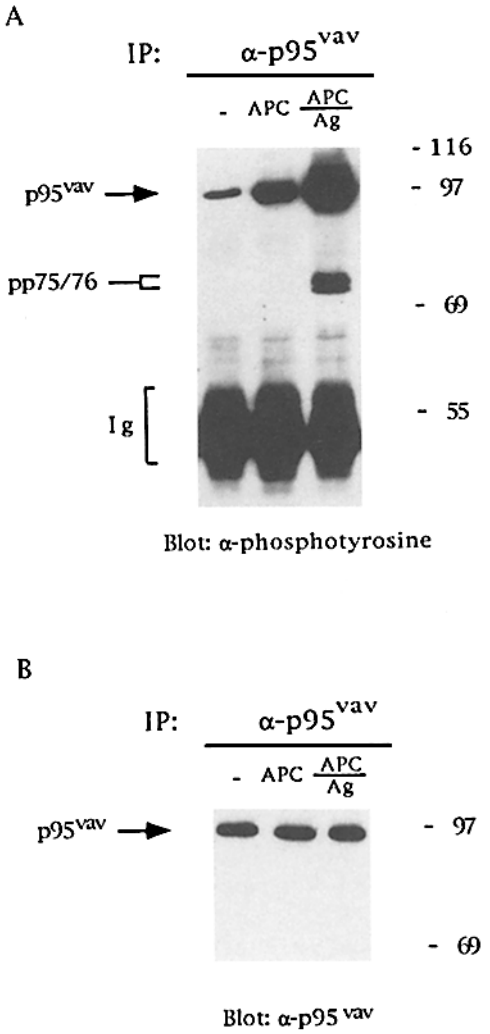

Figure 2. p95^{vav} is tyrosine phosphorylated and associated with a pp75/76 phosphotyrosine protein after TCR engagement by Ag–MHC. Lysates from 10^7 T.AL8.1 cells unstimulated (−) or activated for 5 min with L625.7 cells (*APC*) or antigen-pulsed L625.7 cells (*APC/Ag*) were immunoprecipitated (*IP*) with anti-p95^{vav} Ab. Samples were analyzed on an 8% SDS–polyacrylamide gel by antiphosphotyrosine immunoblotting (*A*) or reprobed with anti-p95^{vav} Ab (*B*).

anti–ZAP-70 immunoblotting (data not shown), and of 36/38-kD protein (*pp36/38*) visible especially in T.AL8.1 cells. These experiments were done using cell suspensions. However, similar results were obtained when Ag-pulsed adherent L625.7 cells were used, and the T cell hybridomas were rapidly detached after stimulation to ensure that the observed phosphotyrosine proteins belonged to the hybridomas (data not shown). The pattern of tyrosine phosphorylation in Ag-stimulated T.AL8.1 cells was similar to that induced by anti-CD3 antibodies (2C11). Nevertheless, the phosphorylation level of pp95 and pp75/76 in Ag-stimulated cells was stronger when compared with that induced by anti-CD3 triggering, whereas the intensity of the other phosphoproteins remained substantially unchanged.

Based on the molecular mass, p95^{vav} could be a likely candidate for the 95-kD protein strongly phosphorylated after Ag stimulation. To test this hypothesis and to identify phosphotyrosine proteins associated to p95^{vav} after TCR stimulation, immunoprecipitates of p95^{vav} were analyzed in antiphosphotyrosine immunoblots. As shown in Fig. 2 *A*, unstimulated T.AL8.1 hybridoma showed a weak basal level of p95^{vav} phosphorylation that increased after stimulation in the presence of L625.7 cells. Tyrosine phosphorylation of p95^{vav} was also observed after incubation of the TCR/CD3-negative parental T cell hybridoma 58$\alpha^-\beta^-$ with L625.7 cells (data not shown), suggesting that other surface molecules different from TCR were responsible for this initial phosphorylation of p95^{vav}. Ag stimulation (Fig. 2 *A*) induced a much higher increase in the level of p95^{vav} phosphorylation and its coprecipitation with the pp75/76 doublet observed in total lysates. Similar results were obtained with T.AL15.3 cells (data not shown). Fig. 2 *B* shows the same blot stripped and reprobed with anti-p95^{vav} Ab to control for equivalent loading of p95^{vav} protein. p95^{vav} could only come from T.AL8.1 cells, as no p95^{vav} was revealed by anti-p95^{vav} immunoblotting in L625.7 cells (data not shown). Thus, TCR triggering by Ag–MHC molecules induced the association of p95^{vav} with tyrosine phosphorylated pp75/76. Although others have reported coimmunoprecipitation of p95^{vav} with ZAP-70 in TCR stimulated Jurkat cells (15), we did not observe this association in our system even after longer exposure of the immunoblots. However, the level of phosphorylated ZAP-70 (Fig. 1) in our cells was in general weaker than in Jurkat cells (data not shown), perhaps explaining the failure to observe this association.

The pp75/76 Associated with p95^{vav} in Ag-stimulated T Cell Hybridomas is SLP-76. One of several proteins tyrosine phosphorylated in Jurkat cells after TCR stimulation is SLP-76, an SH2-containing 76-kD protein expressed exclusively in hematopoietic cells (18). Recent data suggest that SLP-76 may exert an important role in T cell activation (21). SLP-76 contains a conserved tyrosine-containing motif (YE(S/P)P) repeated three times in the NH_2-terminal moiety of the protein (18). Since this motif represents a putative binding sequence for the SH2 of p95^{vav} (15, 24), SLP-76 could be a good candidate for the pp75/76 phosphoprotein interacting with p95^{vav} in an SH2-mediated fashion. To test this possibility, lysates of unstimulated or Ag-activated T.AL8.1 cells were immunoprecipitated with anti-p95^{vav} or with anti–mouse SLP-76 Abs. As shown in Fig. 3 *A* (lane *6*), Ag stimulation induced a strong tyrosine phosphorylation of SLP-76, which appeared as a doublet likely to represent two phosphorylated forms of the protein. Furthermore, SLP-76 comigrated with the pp75/76 phosphotyrosine doublet, which coprecipitated with p95^{vav} after activation (Fig. 3 *A*, lane *3*). A prolonged exposure of the blot corresponding to anti–SLP-76 immunoprecipitates from Ag-activated cells (Fig. 3 *A*, lane *7*) revealed the presence of a 95-kD phosphotyrosine protein with identical electrophoresis mobility to p95^{vav}. SLP-76 was not detected in L625.7 cells by anti–SLP-76 immunoblotting (data not shown). To formally prove the identity of the pp75/76 protein, we performed anti–SLP-76 reimmunoprecipitations from anti-p95^{vav} precipitates (Fig. 3 *B*). Indeed, the pp75/76 doublet associated with p95^{vav} was im-

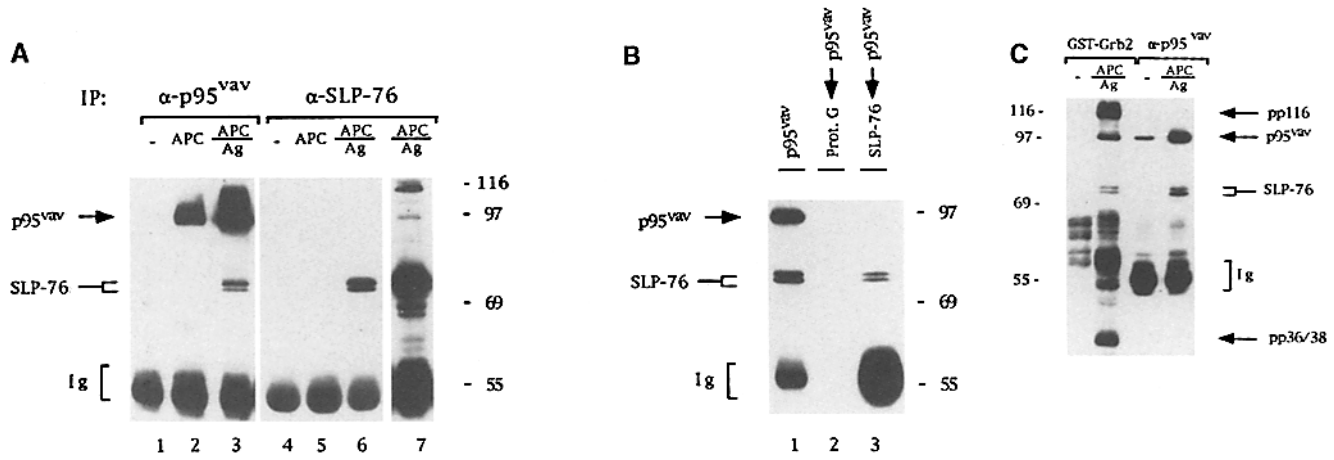

Figure 3. Tyrosine phosphorylated SLP-76 coprecipitates with p95^{vav} in Ag-stimulated cells. (*A*) Immunoprecipitates with anti-p95^{vav} (lanes *1–3*) or anti–SLP-76 Abs (lanes *4–7*) from lysates of 10^7 T.AL8.1 cells unstimulated (−) or activated with L625.7 cells (*APC*) or antigen-pulsed L625.7 cells (*APC/Ag*) were analyzed by antiphosphotyrosine immunoblotting. Films were exposed for 2 min (lanes *1–3* and *7*) or 10 s (lanes *4–6*). (*B*) Lysates were prepared from 4×10^7 T.AL8.1 cells after stimulation with peptide-pulsed L625.7 cells (*APC/Ag*) and immunoprecipitated with anti-p95^{vav} Ab. One half of the precipitates was eluted by boiling in sample buffer (lane *1*), and the second half was subjected to reimmunoprecipitation by anti–SLP-76 Ab (lane *3*) after preclearing with beads of protein G (*Prot.-G*, lane *2*). Phosphorylated proteins were analyzed by antiphosphotyrosine immunoblotting. (*C*) Lysates from 10^7 cells unstimulated (−) or activated with Ag-pulsed L625.7 cells (*APC/Ag*) were incubated with beads coupled with GST–Grb2 fusion protein or immunoprecipitated with anti-p95^{vav} and analyzed as in *A*.

munoprecipitated by anti–SLP-76 antiserum (Fig. 3 *B*, lane *3* vs. lane *1*). No tyrosine phosphorylated proteins were associated with the control protein G beads used in the reimmunoprecipitation experiments (Fig. 3 *B*, lane *2*). Altogether, these results indicate that, after Ag stimulation, p95^{vav} coprecipitates with tyrosine phosphorylated SLP-76. The reason why p95^{vav} was weakly detectable in anti–SLP-76 precipitates could be due to the fact that the anti–SLP-76 antiserum was raised against a large portion of the protein, and it may in part interfere with p95^{vav}–SLP-76 association.

It has been previously reported that in both human T lymphocytes and T cell lines, SLP-76 associated with the SH3 domain of the adapter molecule Grb2 (18–20). A similar interaction has been recently described between p95^{vav} and Grb2 through dimerization of the SH3 domains of each of the two proteins (25). We were therefore interested in verifying whether phosphorylated SLP-76 interacted with Grb2 in Ag-stimulated T cell hybridomas and formed a complex containing p95^{vav}. To this end, a GST–Grb2 fusion protein was used to bind phosphoproteins from the lysate of unstimulated or Ag-activated T.AL8.1 cells. As shown in Fig. 3 *C*, Ag stimulation resulted in the appearance of phosphotyrosine proteins with molecular masses of 116, 95, 75/76, and 36/38 kD that associated with GST–Grb2 as previously reported (18). In particular, the 95- and 75/76-kD phosphotyrosine proteins precipitated by GST–Grb2 possessed identical electrophoresis mobility to p95^{vav} and the associated SLP-76. These data are consistent with reports that identified p95^{vav} and SLP-76 as Grb2-associated proteins (21, 25).

Evidence That the Interaction between p95^{vav} and SLP-76 Is SH2 Mediated. The observation that tyrosine-phosphorylated SLP-76 coprecipitated with p95^{vav}, and the presence of putative binding sites for the SH2 domain of p95^{vav} in SLP-76 (15, 18) suggested to us that p95^{vav}–SLP-76 association would be SH2 mediated. To verify this hypothesis, we investigated whether a tyrosine-phosphorylated peptide could dissociate the p95^{vav}–SLP-76 complex formed in vivo. The competitor peptide contained the YESP sequence that has been described to block the interaction between the SH2 domain of p95^{vav} and ZAP-70 (15). Ag-activated T cell hybridomas were lysed in the absence or presence of unphosphorylated or tyrosine-phosphorylated peptide, and antiphosphotyrosine immunoblots were performed on p95^{vav} immunoprecipitates. As shown in Fig. 4 *A*, the phosphorylated (*Y*ESP*) but not the unphosphorylated (*YESP*) version of the p95^{vav}–SH2 domain competitor peptide was able to inhibit the association between p95^{vav} and SLP-76. A detectable inhibition was also seen at 10-fold lower peptide concentration (data not shown). Fig. 4 *B* shows the same blot reprobed with anti-p95^{vav} antibody to control for the presence of equivalent quantity of p95^{vav} in all immunoprecipitates. Taken together, these results provide evidence that the interaction between p95^{vav} and SLP-76 is SH2 mediated. A recent report by Motto et al. (21) demonstrated that the SLP-76 SH2 domain did not bind to p95^{vav}, but rather to phosphoproteins of 130 and 62 kD. In the light of these data, it is likely that after activation, p95^{vav} interacts via its SH2 domain with tyrosine-phosphorylated SLP-76, not vice versa.

These findings, indicating that p95^{vav} associates in vivo with tyrosine-phosphorylated SLP-76 after TCR ligation by Ag–MHC molecules, may contribute toward understanding the functions of the two molecules in TCR-mediated signaling.

Wu et al. (10) have demonstrated that overexpression of p95^{vav} in Jurkat cells enhanced TCR-mediated signaling leading to NFAT activation, known to depend on both

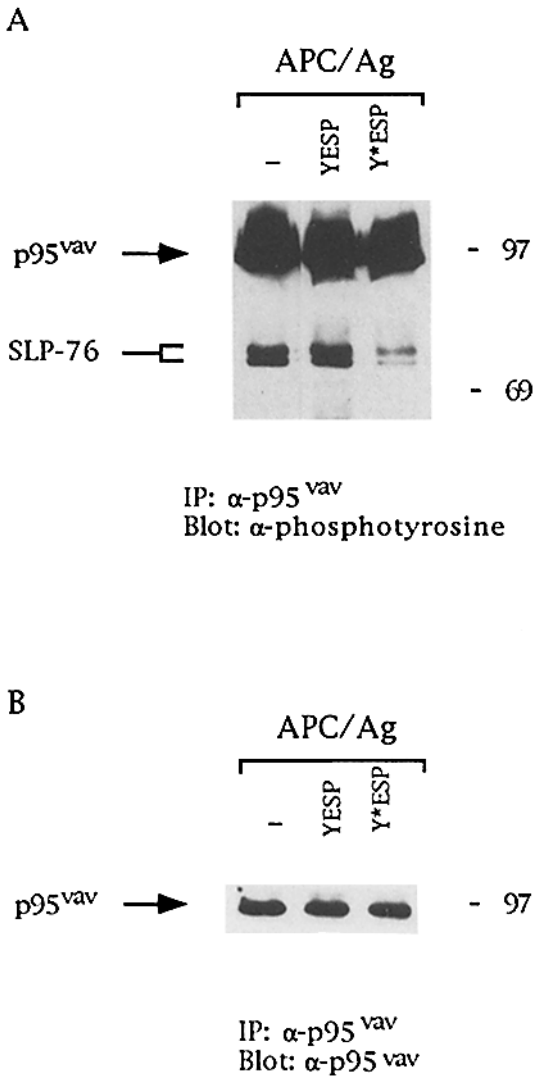

Figure 4. Inhibition of p95^{vav}–SLP-76 interaction by the p95^{vav}–SH2 domain competitor phosphopeptide. 10^7 T.AL8.1 cells activated for 5 min with Ag-pulsed L625.7 cells (*APC/Ag*) were lysed in the presence of 500 μM unphosphorylated or tyrosine-phosphorylated peptide TSVYESPYS (*YESP*); * indicates the presence of a phosphate group. Anti-p95^{vav} immunoprecipitates were analyzed by 8% SDS-PAGE and immunoblotted with antiphosphotyrosine mAb (*A*) or reprobed with anti-p95^{vav} Ab (*B*).

Ras and calcineurin pathways. However, the same report showed that p95^{vav}-dependent stimulation of NFAT was not mimicked by activated p21^{ras}, suggesting that p95^{vav} may control a parallel pathway. This is consistent with data indicating that p95^{vav} is not a GEF for p21^{ras} but rather for other small GTP-binding proteins such as Rho/Rac (13, 14). In this context it is interesting to note that recent results from Genot et al. (26) indicate that in T cells Rac-1 can stimulate the formation of AP-1, which is a component of the active NFAT complex and suggests the existence of pathways parallel to Ras required for NFAT activation. The specific role of Rho in T cells is not known, but Rho has been recently described to control the integrin-mediated adhesion in leukocytes (27). Taken together, these results suggest that p95^{vav} could activate a member of these Rho/Rac proteins and potentiate TCR-mediated signaling.

Similarly to p95^{vav}, SLP-76 overexpression in Jurkat cells enhances TCR-mediated NFAT activity (21). It seems reasonable to hypothesize that the interaction between p95^{vav} and SLP-76 described here forms the basis for the observations that both proteins independently contribute to the same signaling pathway. The SLP-76 sequence did not reveal the presence of catalytic domains (18). However, SLP-76 contains a proline-rich region that has been recently identified as being responsible for the interaction with the SH3 domain of Grb2 and an SH2 domain that interacts specifically with tyrosine phosphorylated proteins of 62 and 130 kD in TCR-activated Jurkat cells (21). Moreover, a tyrosine-containing motif, likely to be involved in the interaction with SH2 containing proteins, is repeated three times in SLP-76. Our data suggest that the tyrosine phosphorylation of these motifs may be responsible for the physical association of SLP-76 with p95^{vav} after TCR stimulation. Furthermore, using a GST–Grb2 fusion protein, we observed the formation in vitro of a complex between Grb2 and proteins possessing electrophoretic mobility identical to p95^{vav}, SLP-76, and a pp36/38 protein. The latter has been described as a membrane-bound protein that is phosphorylated after TCR stimulation and is able to associate with the SH2 domain of Grb2 (28, 29). These data raise the interesting possibility that Grb2, associated via its SH2 domain with pp36/38, binds through the SH3 domain to SLP-76, which in turn, acting as an adapter, facilitates the recruitment of p95^{vav} to the membrane. p95^{vav} may then contribute to the activation of Rho/Rac proteins.

Our observations that an initial phosphorylation of p95^{vav} was seen when the T cell hybridomas were incubated with APC in the absence of Ag and was TCR independent (data not shown) suggest that this event may be mediated by other surface receptors. A possible candidate may be CD28, whose stimulation by B7-1/B7-2 has been shown to induce p95^{vav} phosphorylation (30).

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