Aggregation of the high affinity receptor for IgE (FceRI) on the mucosal mast cell line, RBL-2H3, results in the rapid and persistent tyrosine phosphorylation of Vav. Immunoprecipitation of Vav from activated cells revealed co-immunoprecipitated phosphoproteins of molecular weights identical to the FceRI β and γ chains, and the former was reactive with antibody to the FceRI β chain. Conversely, Western blots revealed the presence of p95 Vav in FceRI immunoprecipitates. The association of Vav with of Grb2 with the receptor was found to be regulated by aggregation of the receptor, and the interaction of Vav with the FceRI was localized to the γ chain. To gain insight on the signaling pathway in which Vav participates, we investigated the in vivo associations of Vav with other molecules. A reducible chemical cross-linking agent was used to covalently maintain protein interactions under nonreducing conditions. A fraction of Vav increased in mass to form a complex of >300 kDa in molecular mass. Under reducing conditions the cross-linked Vav immunoprecipitates showed the presence of Grb2, Raf-1, and p42\textsuperscript{mapk} (ERK2). In vitro kinase assays of Raf-1 activity associated with Vav revealed that this complex had an activity greater than that of Raf-1 derived from nonactivated cells, and aggregation of the FceRI did not modulate this activity. In contrast, aggregation of the FceRI increased the total Raf-1 activity by 2–5-fold. These results demonstrate that Vav associates constitutively with components of the mitogen-activated protein kinase pathway to form an active multimeric signaling complex whose in vivo activity and associations may be directed by aggregation of the FceRI. The findings of this study may also be relevant to other members of the immune recognition receptor family that share the T-cell antigen receptor βγ chains.

Antigen triggering of mast cells and basophils via cell-bound immunoglobulin E (IgE) stimulates the activation of serine/threonine and tyrosine kinases leading to phosphorylation of multiple cellular substrates (1, 2). A critical step in the antigen activation of mast cells is the phosphorylation of the high affinity receptor for IgE (FceRI)\textsuperscript{+} on both the β and γ chains of the tetrameric receptor (αβγγ) (3, 4). A number of molecules that participate either in the phosphorylation of the receptor or of other substrates, in response to aggregation of the receptor, have been identified. These include p56\textsuperscript{lck} (Lyn), pp60\textsuperscript{src} (Src), p72\textsuperscript{Syk} (Syk), Bruton’s tyrosine kinase, and PKC-δ (5–8). Evidence has been presented for the recruitment of some of these molecules to the receptor, in response to the phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM, 9) present on the β and γ chains of this receptor. Benhamou and colleagues (10) reported the association of the Syk, which is critical for its ability to recruit molecules that link receptors to downstream effectors (12).

In the mast cell one potential downstream effector is Vav, a hematopoietic cell-specific protein that exhibits high homology to guanine nucleotide exchange factor (GEF) proteins (13). Vav is expressed as a p95 proto-oncogene product or a p85 oncogenic product (14). The homology of the Vav with other members of the GEF family suggests that Vav may regulate guanine nucleotide exchange on the Rho family of GTP-binding proteins (13). However, in T and B lymphocytes Vav was reported to exhibit an increased GDP exchange activity directed toward Ras (15, 16). Subsequent studies suggest that Vav does not exhibit direct exchange activity toward Ras but requires Ras activity to demonstrate its phenotype (17). Whether Vav has intrinsic GEF activity toward Ras, or any other GTP-binding protein, or if its association with a GEF expressed in hematopoietic cells (18) accounts for the exhibited GEF activity is still unclear. Nevertheless, Vav has been shown to form a complex with a variety of proteins including ZAP 70, Grb2, and the RNA-binding protein, heterogeneous nuclear ribonucleoprotein K (19–21). These associations may be important to its role in development of T and B cells as well as in T-cell antigen receptor signaling (22, 23, 45). In contrast, other hematopoietic cells including cells of mast cell lineage appear to develop normally from progenitor cells that lack Vav (24). Therefore it is possible that Vav might serve a different role in mast cells or, alternatively, other proteins might compensate for Vav signaling in these cells.
Tyrosine phosphorylation of p95 Vav in response to aggregation of the FcεRI (25) suggests a role for this protein in receptor-mediated signal transduction in mast cells. The phosphorylation of Vav has been linked to the expression of Syk kinase activity in the mucosal mast cell model, RBL-2H3 (26). Since Syk is recruited to the FcεRI upon its aggregation (10), we have investigated whether a potential downstream effector such as Vav could associate with the receptor and might this association be regulated by aggregation of the receptor. Furthermore, to gain insights as to the signaling pathway in which Vav participates in mast cells, we analyzed which proteins complex with Vav in vivo and determined if kinase activity is associated with Vav.

MATERIALS AND METHODS

Immunoglobulins—Anti-dinitrophenyl (DNP)-specific mouse monoclonal IgE (27) was purified as described previously (28). Goat anti-mouse IgE was purchased from ICN (Costa Mesa, CA). The monoclonal antibody to the FcεRI β chain (mAb β JIR) was purified as described (29). Rabbit polyclonal antibodies to Vav, Lyn, Grb2, and ZAP70 were purchased from Santa Cruz Biotech or Transduction Laboratories, respectively. A mouse monoclonal antibody to Vav raised to a glutathione S-transferase-fusion protein of full-length human Vav was purchased from Upstate Biotechnologies, Lake Placid, NY. Rabbit polyclonal antibodies to Vav, raised to either a peptide corresponding to amino acids 926–941 of human Vav or to fragment of human Vav (amino acids 620–797), were purchased from Santa Cruz Biotech or Transduction Laboratories, respectively. A mouse monoclonal antibody to Lyn raised to a glutathione S-transferase-fusion protein of full-length human Lyn was purchased from Santa Cruz Biotech. Rabbit antiserum to Syk was kindly provided by Dr. R. Siragianian (NIDR, NIH). Purified normal rabbit or goat IgG was purchased from Jackson Immunoresearch, West Grove, PA.

Cells and Activation—The 2H3 subline of RBL cells was cultured as a monolayer in stationary flasks essentially as described (30). For experiments the cells were sensitized with 125I-labeled or unlabeled dinitrophenyl-specific mouse monoclonal IgE (as indicated) at 2.5 μg/ml for 5 × 10⁶ cells/ml for 1 h at room temperature or by addition directly to the culture flask of 1 μg/ml overnight at 37°C. Cells in modified Tyrode’s buffer (31) were activated by incubation with 0.4–1.0 μg/ml dinitrophenylated bovine serum albumin (DNP-BSA) at 37°C for 3 min or for the indicated time. Activation was stopped by the immediate addition of a 2× ice-cold lysis buffer (see below). For some experiments cells in media were activated as above, and activation was quenched by addition of an ice-cold phosphate-buffered saline, pH 7.4, cells were washed in ice-cold phosphate-buffered saline and lysed.

Cell Solubilization and Immunoprecipitation—For studies to analyze the association of Vav with receptor, 0.5–5.0 × 10⁶ cells previously labeled with 125I-IgE were solubilized at a final concentration of 0.0825% Triton X-100 (Research Products International, Mount Prospect, IL) for 5 × 10⁶ cells. All solutions contained protease and phosphatase inhibitors as described (32). Solubilization was for 1 h at 4°C which allowed for recovery of greater than 95% of the nonaggregated and DNP-BSA-aggregated receptor. Soluble cell lysates were recovered after a 15-min microcentrifugation at 13,000 × g. Lysates were treated with protein G or protein A-Sepharose for 1 h at 4°C. The cleared lysates were subsequently immunoprecipitated with goat anti-mouse IgG (10–15 μl) or with 10 μg of rabbit antibodies to Vav, Lyn, Grb2, ZAP70, and rabbit IgG for each individual sample. Protein G or protein A-Sepharose was added after 2 h, and incubation was continued overnight at 4°C. Recovered immunoprecipitates were washed 6× in 0.1% Triton X-100 containing protease and phosphatase inhibitors. 125I-IgE receptors recovered in the immunoprecipitates of Vav, Lyn, Grb2, and ZAP70 were quantitated by counting in a Packard Minaxi Auto Gamma 5000 (Downers Grove, IL). Samples of the immunoprecipitated proteins were also solubilized in Tris glycine-SDS sample buffer and proteins resolved by SDS-PAGE.

For analysis of Vav-associated proteins, cells (2 × 10⁷) were solubilized in 0.3% Triton X-100 containing protease and phosphatase inhibitors. Vav, ERK2, and Syk were immunoprecipitated by addition of 10 μg of anti-Vav or anti-ERK or 40 μg of an IgG fraction of anti-Syk followed by protein A-Sepharose. The immunoprecipitates were washed 5 times in 0.5% Triton X-100 and 1 time in 0.01% Triton X-100 and solubilized in Tris glycine-SDS sample buffer. Proteins were resolved and immunoblotted as described below. For chemical cross-linking experiments nonactivated or activated cells (2 × 10⁷) were solubilized in cross-linking buffer (25 mM HEPES, pH 8.0, 5 mM KCl, 119.4 mM NaCl, 5.6 mM glucose, 1 mM MgCl₂, and 0.5 mM CaCl₂) containing 1% Nonidet P-40 (Nonidet P-40) with protease and phosphatase inhibitors. The soluble cell lysates were incubated, for 30 min on ice, with 2 μM DTSSP (3,3′-dithiobis(sulfosuccinimidyl propionate), pH 8.0, a concentration determined to cause effective cross-linking and maintain antibody reactivity. Reaction was stopped by addition of 40 μM glycine and further incubation on ice for 20 min. Immunoprecipitation was as described above and recovered pellets were washed 5 times with 0.1% PFA buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS) followed by 1 time in 10 mM Tris-HCl, pH 7.4. The antibodies used to detect cross-linked proteins were tested for their ability to recognize the protein in chemically cross-linked cell lysates. Glycathione S-transferase-fusion proteins were purified and used as described previously (8).

Affinity Purification of Vav—Affinity-purified Vav was obtained from cells solubilized in 0.5–1% Triton X-100 or Nonidet P-40 containing protease and phosphatase inhibitors. In some experiments nuclei were removed by microcentrifugation, and supernatants were applied to a Vav affinity column (Santa Cruz Biotech, Santa Cruz, CA) at 4°C. Columns were washed 3 times with phosphate-buffered saline using 20-fold bed volume per wash. Proteins bound were eluted with 100 mM CAPS buffer, pH 11.7, and immediately neutralized to pH 7.4 with 1 N HCl.

Gel Electrophoresis and Western Blots—Proteins were resolved on 8, 10, and 4–12% Tris glycine gels under reducing conditions, unless otherwise stated, and transferred (as described) to 0.45-μm nitrocellulose membranes. Membranes were blocked and phosphorytousine-containing proteins and Vav (yeast 0.5 μg/ml) or Vav (polyclonal or monoclonal antibody specific to Vav was used at a dilution of 1:1667 or 1:1000, respectively. In some experiments the F(ab)₂ fragment of donkey anti-rabbit or sheep anti-mouse Ig conjugated with horseradish peroxidase (Amersham Corp.) was used at a 1:12,500 dilution as a secondary antibody. In other experiments a biotinylated mouse monoclonal anti-rabbit Ig was used as a secondary antibody with subsequent addition of horseradish peroxidase-conjugated Extravidin (Sigma). Visualization of reactive proteins was by enhanced chemiluminescence (Amersham Corp.). Relative quantitation was by densitometric analysis of exposures in the linear range using a Molecular Dynamics densitometer and ImageQuant software for analysis (Sunnyvale, CA).

In Vitro Kinase Assays—Cells were incubated for 8 h in serum-free or serum-containing media prior to stimulation to obtain Vav from quiescent or nonquiescent cells. Antigen stimulation, lysis, chemical cross-linking, and immunoprecipitation are described above. Raf-1 and Vav immunoprecipitates were subjected to three washes in 1% Nonidet P-40 (see above). Thirty microliters of Raf kinase assay buffer (0.2 mM ATP, 30 mM MgCl₂, 2 mM MoCl₃, 40 mM sodium β-glycerophosphate, 0.2 mM sodium orthovanadate, 2 μM okadaic acid, 0.2% β-mercaptoethanol) was added to each immunoprecipitate along with 1 μg of purified human recombinant MEK1. Incubation was at 30°C for 10 min followed by the sequential addition of 20 μg of [γ-32P]ATP and 1 μg of purified ERK2 kinase-inactive mutant (K52R) for a final volume of 50 μl and a subsequent incubation of 2 min at 30°C. Reactions were stopped by addition of boiling 5-fold-concentrated SDS sample buffer, and proteins were resolved by 10% Tris glycine-PAGE. Proteins were transferred to nitrocellulose, as above, and probed for the presence of Raf-1 and subsequently exposed to autoradiography. We did not observe significant differences in Raf-1 activity derived from quiescent or nonquiescent cells.

RESULTS

FcεRI-mediated Regulation of Vav and Grb2-associated Tyrosine Phosphoproteins—Aggregation of the FcεRI on RBL-2H3 cells results in the phosphorylation of the receptor and associated proteins (Fig. 1, IgE). To determine whether Vav and the Vav-associated Grb2 (20) might complex to receptor in response to its aggregation, we first analyzed the apparent molecular masses of tyrosine-phosphorylated proteins that immunoprecipitated with Vav or Grb2 in nonstimulated and antigen-stimulated cells. Fig. 1 shows that in immunoprecipitates of Vav derived from resting cells, Vav is tyrosine-phosphorylated. Tyrosine phosphorylation of Vav is increased upon aggregation of the receptor. Other tyrosine-phosphorylated proteins were also detected in immunoprecipitates of Vav. Two such
proteins at 18 and 30 kDa had the identical molecular mass of the FcRI β chain and γ chain dimer, respectively, under nonreducing conditions. Previous studies have shown that Grb2 associates with multiple tyrosine-phosphorylated proteins in response to aggregation of the FcRI (33). Consistent with previous results immunoprecipitates of Grb2 revealed multiple tyrosine-phosphorylated proteins associated with Grb2 prior to and after the aggregation of the receptor (33). Aggregation of the receptor induced only a slight increase in the tyrosine phosphorylation of Shc under nonquiescent conditions (33), but new phosphoproteins at molecular masses of 18, 30, 33, and 75 kDa were observed (Fig. 1). To determine if the 18-kDa band in immunoprecipitates of Vav and Grb2 had the characteristics of the disulfide-linked γ chain dimer of the FcRI, immunoprecipitates were subsequently analyzed on SDS-PAGE under reducing conditions. Almost complete loss of the bands ranging from 14 to 20 kDa was observed in immunoprecipitates of Vav and significant loss of the phosphoproteins of this molecular mass range was observed in immunoprecipitates of Grb2 (data not shown).

**Kinetics of Tyrosine Phosphorylation of Vav and of the FcRI β and γ Chains**—The association of Vav with FcRI might be dependent on the tyrosine phosphorylation of Vav or of the receptor. We investigated the kinetics of phosphorylation of Vav and compared this to phosphorylation of the FcRI β and γ chain. Fig. 2 shows that the tyrosine phosphorylation of p95 Vav is rapid with a maximum reached between 1 and 3 min of aggregation of the receptor. This mimics the early phosphorylation kinetics of the FcRI β and γ chains, but dephosphorylation of the receptor is rapid while phosphorylation of Vav is maintained through 27 min of activation. All further experiments to study the Vav and Grb2 interactions with receptor or Vav interactions with other proteins were done 2 min after addition of the aggregating antigen (DNP-BSA).

**Association of Vav with the FcRI γ Chain**—The possible association of Vav with the FcRI was further investigated by isolation of the receptor or Vav under conditions that maintain receptor subunit association (8, 32). Immunoprecipitates (IP) of Vav or FcRI were resolved on SDS-PAGE, transferred to nitrocellulose, and probed with antibody to phosphotyrosine (PY). This was followed by reprobing of stripped immunoblots with antibody to Vav or to FcRI β chain. Densitometric analysis of the levels of tyrosine-phosphorylated Vav (●), FcRI β (■), and γ chains (▲) is shown. Data are normalized to the relative amounts of Vav or FcRI β chain detected on immunoblots. Data shown in graph were compiled from three experiments; one representative immunoblot is shown.
Cellular Vav was associated with the FceRI. This might explain the inability to detect tyrosine-phosphorylated Vav in association with receptor. Furthermore, preliminary experiments suggest that only a small fraction of Vav (<20%) is tyrosine-phosphorylated which would further decrease the sensitivity of phosphotyrosine detection. In contrast when Vav was immunoprecipitated from activated cells, 2-3% of the receptor, as detected by immunoprecipitation of cell surface-bound and radio-labeled IgE, was co-immunoprecipitated with Vav (Fig. 3B). The extent of this co-immunoprecipitation was comparable with the co-immunoprecipitation of receptor with antibody to the receptor-associated p53/56\textsuperscript{cyt} kinase (Fig. 3B), although without use of chemical cross-linking agents the latter association was variable. To determine which subunit of the Fc\textsubscript{e}R\textsubscript{I} might interact with Vav, glutathione S-transferase (GST)-fusion proteins containing the cytoplasmic domains of Fc\textsubscript{e}R\textsubscript{I} β and γ chains were reacted with cell lysates derived from both nonactivated and activated RBL-2H3 cells. Fig. 3C shows a 95-kDa band reactive with antibody to Vav associated with the C-terminal domain of the γ chain. This association was specific since associations with GST or with other GST-fusion proteins were not observed, and probing of the GST-fusion proteins with antibody to PKC-δ revealed this isozyme was associated with the GST-β construct as described previously (data not shown, 8). An increased association of Vav with GST-γ could not be detected with Vav derived from either nonactivated or activated cells. Both polyclonal and monoclonal antibody to Vav recognized this GST-γ-associated protein (data not shown). Interestingly, only the 95-kDa protein reactive with both polyclonal and monoclonal antibodies to Vav was observed with the GST-γ-fusion protein or with receptor, although a Vav antibody-reactive protein of 85 kDa (which appears to be a proteolytic product of Vav; data not shown) is detected in cell lysates.

**Regulation of Fc\textsubscript{e}R\textsubscript{I} Association with Vav and Grb2**—The association of the Fc\textsubscript{e}R\textsubscript{I} with Vav or Grb2 is regulated by aggregation of the receptor (Fig. 4). Immunoprecipitates of Vav or of Grb2 isolated under conditions that maintain the intact tetrameric structure of the receptor showed the presence of co-immunoprecipitated receptor, as detected by monoclonal antibody to Fc\textsubscript{e}R\textsubscript{I} β chain. In close agreement with the data presented in the previous section, relative quantitation of immunoblots showed that approximately 2% of the receptors were co-immunoprecipitated with both Vav and Grb2 (Fig. 4A) after aggregation of the receptor. While these results suggest that receptors interact with Vav or Grb2, only after aggregation of the receptors, long exposures of the immunoblots revealed the presence of the β chain in the immunoprecipitates derived from nonaggregated cells (data not shown). This is consistent with the data shown in Fig. 3A. Nevertheless, an enhancement of the association is seen upon aggregation of the receptor, and kinetic analysis of receptor association with Vav revealed that peak association occurs between 1 and 3 min, a time when the phosphorylation of the receptor is maximal (Fig. 4B and Fig. 2). Collectively, the data suggest that a 2–4-fold enhancement of Vav in association with the receptor is mediated by the aggregation of the Fc\textsubscript{e}R\textsubscript{I}.

**Raf-1, MEK, and ERK Co-immunoprecipitate with Vav**—Prior studies implicate Vav as a component of the Syk-dependent activation of MAP kinase activity (26). To determine if the association of Vav with the Fc\textsubscript{e}R\textsubscript{I} might link the receptor to the activation of the MAP kinase pathway, we analyzed whether Vav might associate with proteins that lead to MAP kinase activation in mast cells (34). Nonactivated cells were solubilized using 0.5% Triton X-100 or 1.0% Nonidet P-40 to obtain maximal solubility of cellular proteins. Fig. 5A shows that immunoprecipitation of Vav under these conditions led to the specific co-immunoprecipitation of Raf-1 and p42/p44\textsuperscript{mapk}. Relative quantitation of immunoblots suggests that approximately 3% of cellular Raf-1 is complexed with Vav. Immunoprecipitation of p42/p44\textsuperscript{mapk} or of Syk revealed that less than 1% of cellular Raf-1 co-immunoprecipitated with these proteins (Fig. 5A). All detected associations appeared to be specific since co-immunoprecipitation was not observed in IgG control immunoprecipitates (Fig. 5A), and other component proteins of the MAP kinase pathway (Sos and Ras) were not detected in immunoprecipitates of Vav (Table I). Immunoprecipitates of Vav...
were also found to contain the p42 (ERK2) and p44 (ERK1) forms of MAP kinases (Fig. 5A). The predominant form associated was ERK2 of which greater than 1% of total cellular ERK2 was co-immunoprecipitated with Vav. Immunoprecipitation of Syk also revealed the presence of ERK1 and ERK2 isoforms although to a lesser extent than that observed with Vav. To determine if MEK1, the kinase of dual specificity (35) that activates ERKs, might also be found as a component of the Vav-associated complexes, a summary of Vav-associated proteins is presented in Table I.

**Table I**

Co-immunoprecipitation of Vav-associated proteins

Summary of Vav-associated proteins as determined by varying protocols of Vav isolation described under "Materials and Methods." Experiments done at least twice are included. ND is not determined. Vav was isolated by immunoprecipitation (IP), affinity chromatography (AP), or from chemical cross-linking experiments using DTSSP to covalently maintain protein-protein interactions (X-link).

| Protein     | IP | AP | X-link | % Associated |
|-------------|----|----|--------|--------------|
| Grb2        | +  | +  | +      | >1           |
| Raf-1       | +  | +  | +      | ~3           |
| ERK1        | +  | +  | +      | >1           |
| MEK1        | ND | +  | +      | <1           |
| Pan Ras     | -  | -  | -      | -            |
| PLCγ        | -  | -  | -      | -            |
| Shc         | -  | ND | -      | -            |
| Sos 1/2     | -  | +  | ND     | ND           |
| Syk         | +  | +  | +      | ND           |
| Rac 1/2     | -  | -  | -      | -            |
| Ras-GAP     | -  | -  | -      | -            |
| RhoA        | -  | -  | -      | -            |
| RhoB        | -  | -  | -      | -            |
| Rho-GAP     | -  | -  | -      | -            |

* The percent of total cellular protein associated with Vav as determined by relative quantitation of immunoblots. Values shown as >1 did not exceed 1.5%, and values shown as <1 were between 0.3 and 0.6% except for Syk where no value could be determined. Significance is given in terms of values for other in vivo interactions (11, 37).

* The co-immunoprecipitation of ERK1 and -2 (p44 and p42) was found in Vav immunoprecipitates isolated by IP and AP. In X-link Vav immunoprecipitates only the p42 isoform (ERK2) could be reproducibly detected.

* Immunoprecipitation of Syk and immunoblotting of the immunoprecipitates with antibody to Vav. This approach was chosen due to the limited sensitivity of the antibody to Syk on Western blots.

Fig. 5. Association of Raf-1, MEK1, and ERK with Vav. A, cells (2.0 × 10⁷) were solubilized in 0.5% Triton X-100 and ERKs, Vav and Syk, were immunoprecipitated (IP). Control immunoprecipitations were done with rabbit IgG. Proteins were resolved on SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the indicated antibody. One representative of three (Raf-1) or four (ERK1 and ERK2) experiments is shown. CE indicates a cell lysate equivalent of 2 × 10⁵. B, cells (8.0 × 10⁷) were solubilized in 1% Nonidet P-40, and Vav was affinity-purified by column chromatography and eluted as recommended by the manufacturer (Santa Cruz Biotech, Santa Cruz, CA). A cell equivalent of 4 × 10⁶ of eluate derived from nonactivated (−) or from DNP-BSA activated (+) cells (1 μg/ml) was resolved on SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibody to MEK1. CE indicates a cell lysate equivalent of 1 × 10⁶. One representative of two experiments is shown.

Tyrosine phosphorylation of p95 Vav was found to contain the p42 (ERK2) and p44 (ERK1) forms of MAP kinases (Fig. 5A). The predominant form associated was ERK2 of which greater than 1% of total cellular ERK2 was co-immunoprecipitated with Vav. Immunoprecipitation of Syk also revealed the presence of ERK1 and ERK2 isoforms although to a lesser extent than that observed with Vav. To determine if MEK1, the kinase of dual specificity (35) that activates ERKs, might also be found as a component of the Vav-associated complexes, a summary of Vav-associated proteins is presented in Table I. Experiments done at least twice are included. ND is not determined. Vav was isolated by immunoprecipitation (IP), affinity chromatography (AP), or from chemical cross-linking experiments using DTSSP to covalently maintain protein-protein interactions (X-link).

| Protein     | IP | AP | X-link | % Associated |
|-------------|----|----|--------|--------------|
| Grb2        | +  | +  | +      | >1           |
| Raf-1       | +  | +  | +      | ~3           |
| ERK1        | +  | +  | +      | >1           |
| MEK1        | ND | +  | +      | <1           |
| Pan Ras     | -  | -  | -      | -            |
| PLCγ        | -  | -  | -      | -            |
| Shc         | -  | ND | -      | -            |
| Sos 1/2     | -  | +  | ND     | ND           |
| Syk         | +  | +  | +      | ND           |
| Rac 1/2     | -  | -  | -      | -            |
| Ras-GAP     | -  | -  | -      | -            |
| RhoA        | -  | -  | -      | -            |
| RhoB        | -  | -  | -      | -            |
| Rho-GAP     | -  | -  | -      | -            |

* The percent of total cellular protein associated with Vav as determined by relative quantitation of immunoblots. Values shown as >1 did not exceed 1.5%, and values shown as <1 were between 0.3 and 0.6% except for Syk where no value could be determined. Significance is given in terms of values for other in vivo interactions (11, 37).

* The co-immunoprecipitation of ERK1 and -2 (p44 and p42) was found in Vav immunoprecipitates isolated by IP and AP. In X-link Vav immunoprecipitates only the p42 isoform (ERK2) could be reproducibly detected.

* Immunoprecipitation of Syk and immunoblotting of the immunoprecipitates with antibody to Vav. This approach was chosen due to the limited sensitivity of the antibody to Syk on Western blots.

Fig. 4. Regulation of FceRI association with Vav and Grb2. Anti-FceRI-sensitized cells (5 × 10⁶/ml) were activated with (+) 0.4 μg/ml DNP-BSA or not (−) for 2 min. A, cells were solubilized under conditions described in Fig. 2 legend, and receptor, Vav, and Grb2 were immunoprecipitated (IP). Antibody to ZAP 70 was used in control immunoprecipitates. Proteins (1 × 10⁶) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with antibody to FceRI β chain. CE indicates cell lysate equivalent of 8 × 10⁶ cells. One representative of three experiments is shown. B, immunoprecipitates of Vav, isolated as above, from cells stimulated with DNP-BSA for the indicated time. Isolated immunoprecipitates were washed 6 × in 0.01% Triton X-100. Data shown is net, derived from three experiments where control immunoprecipitates were done with antibody to ZAP 70.
Vav-associated protein complex, we affinity-purified Vav and eluted the protein in the absence of purifying antibody (antibody to Vav was covalently cross-linked to the matrix) to avoid masking of detected MEK1 by presence of immunoglobulin heavy chain at the apparent molecular weight of MEKI. Fig. 5B shows that the affinity-purified Vav does contain a component reactive with antibody specific to MEK1 whose association with Vav does not appear to be receptor-regulated. We could not reproduce these results using a chemical cross-linking agent (Table I). However, we cannot be certain that MEK1 was covalently cross-linked to Vav, and therefore, we may have disrupted its association with harsh washing conditions. Nevertheless, the data suggest that components of the MAP kinase pathway are constitutively associated with Vav as a multicomponent signaling complex.

**Chemical Cross-linking of Vav-associated Proteins and Effect of Aggregation of the Receptor**—The constitutive association of components of the MAP kinase pathway with Vav would predict the presence of high molecular weight signaling complexes that might be covalently cross-linked by chemical cross-linking reagents and reactive with antibodies to Vav. Fig. 6A shows that proteins of multiple molecular masses were detected by immunoprecipitation of Vav or in cell lysates reacted with antibody to Vav. In 6% SDS-PAGE, the predominant Vav-reactive components had apparent molecular masses of 84, 105, 115, and 180 kDa with the predominant form at 105 kDa. Polyclonal or monoclonal antibodies to Vav gave virtually identical patterns. Chemical cross-linking of cell lysates with the reducible cross-linking agent DTSSP and resolution of proteins under nonreducing conditions showed a newly generated Vav-reactive component of an apparent molecular mass greater than 300 kDa (Fig. 6A, lysate, lane 3). Concomitantly, a disappearance of the 115- and 180-kDa bands was observed. These bands were restored by running samples under reducing conditions (Fig. 6A, lysate, lane 4). Immunoprecipitated Vav from chemically cross-linked cells resolved under nonreducing conditions also showed a Vav-reactive band of an apparent molecular mass greater than 300 kDa (Fig. 6A, IP, lane 3). These results show that a high molecular weight complex could be isolated with antibody to Vav, and furthermore reduction of the isolated complex restores Vav-reactive components of 115 and 180 kDa. The molecular masses of these components are most consistent with a Vav-Grb2 and a Vav-Grb2-Raf-1 or Syk complex, respectively.

Figs. 1 and 4A show that Grb2 is found in association with FceRI. One possible mechanism for this association might be that Grb2 interacts with Vav as described previously (20) and therefore associates with receptor as part of this multicomponent complex. Fig. 6B shows that Grb2 was found in immunoprecipitates of Vav from nonactivated and activated cells. An increase in Grb2 association with Vav was not detected in immunoprecipitates of Vav from activated cells; however, the
The Vav-associated Protein Complex Is an Active Complex—To assess if Vav is a component of a receptor-activated complex, we investigated if aggregation of the receptor led to increased activity of Vav-associated Raf-1 since this kinase was most abundant in this complex. Fig. 7 shows that aggregation of the FcεRI results in an increase of approximately 5-fold in Raf-1 activity. The activity of Raf-1 present in Vav immunoprecipitates was found to be greater than the activity of Raf-1 from nonactivated cells and did not appear to be modulated by aggregation of the FcεRI (Fig. 7). In some experiments the activity of Vav-associated Raf-1 was 50–70% of the Raf-1 activity in activated cells. Normalizing to the amount of Raf-1 present in Vav immunoprecipitates, Vav-associated Raf-1 is up to 6-fold more active than the remaining Raf-1 prior to aggregation of the FcεRI. This suggests that the Vav-associated multicomponent complex is a constitutively active complex whose activity may be localized by protein-protein interactions in response to the aggregation of the FcεRI.

**DISCUSSION**

The in vitro approach in the study of Vav-associated proteins has revealed the identity of a number of molecules that may be important in Vav-related signaling (19–21, 36). Some of these proteins include Grb2, Nck, c-Crk, She, phosphatidylinositol-3 kinase, phospholipase C-γ1, Ras-GAP, heterogeneous nuclear ribonucleoprotein K, and ZAP 70. Given that Vav may mediate cell-specific protein-protein interactions, we have investigated the in vivo association of Vav with proteins in the mucosal mast cell model, RBL-2H3, as a first step toward elucidating the role of Vav in mast cell signaling. Our findings are 1) that Vav associates with the FcεRI γ chain and this association is regulated by the aggregation of the receptor; 2) that Vav is present in Syk immunoprecipitates and the latter activity was previously shown to be necessary for Vav phosphorylation (26); 3) that a fraction of Vav is part of a multicomponent signaling complex that is constitutively present and active in the mast cell and is comprised of at least Grb2, Raf-1, and ERK2, all components of the MAP kinase pathway, and Grb2 and Vav can be found to associate with the receptor; 4) and that Vav is part of an active complex as demonstrated by the activity of Vav-associated Raf-1 which was found to be greater than Raf-1 activity from nonactivated cells. Our findings provide evidence for the participation of Vav in the activation of the MAP kinase pathway in the mast cell and promote the idea of an active multi-component signaling complex whose activity is directed to the receptor by phosphorylation of the receptor γ chain ITAM (Fig. 8).

However, we did detect low levels of Vav interacting with receptor in nonactivated cells, and Vav was also found to associate with the GST-γ-fusion protein which had no detectable levels of phosphotyrosine. Therefore, it is possible that the interaction of the Vav-containing complex with the receptor

**FIG. 7. Raf-1 kinase activity in immunoprecipitates of Vav and Raf-1.** Immunoprecipitation and in vitro kinase assays were as described under “Materials and Methods.” Briefly, Raf-1 kinase activity was determined by an amplification assay in which the activation of MEK1 by Raf-1 is measured by phosphorylation of the MEK1-specific substrate ERK2. Control immunoprecipitates with rabbit IgG were assayed under identical conditions, and no detectable activity was found. In the absence of MEK1 no phosphorylation of ERK2 was detected. To determine the activity of Raf-1 or of Vav-associated Raf-1 from nonactivated (−) or DNP-BSA-activated (+) cells, the level of phosphorylation was determined by autoradiography, and relative quantification was by densitometric analysis. Activities were normalized to the relative amount of Raf-1 detected in Western blots of the Vav or Raf-1 immunoprecipitates. Data are presented as a fraction of the Raf-1 activity found in activated cells (+) and are compiled from three experiments. ** indicates a significant difference (p < 0.02, paired t test) when compared with Raf-1 activity in nonactivated cells. * indicates a significant difference (p < 0.06, paired t test) when compared with Raf-1 activity in nonactivated cells.

**Association of Vav with Vav was maintained even after stringent washing of noncross-linked samples (see “Materials and Methods”).** Similar results were obtained for the kinase Raf-1 (Fig. 6C). Aggregation of the receptor did not enhance Raf-1 association with Vav, and the association proved to be resistant to stringent wash conditions. Relative quantitation of immunoblots suggested the presence of greater than 1% of the cellular ERK2 in immunoprecipitates of Vav. However, we did detect low levels of Vav interacting with receptor in nonactivated cells, and Vav was also found to associate with the GST-γ-fusion protein which had no detectable levels of phosphotyrosine. Therefore, it is possible that the interaction of the Vav-containing complex with the receptor

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ies to distinct epitopes of ERK2 gave identical results (data not shown). Relative quantitation of immunoblots suggested the presence of greater than 1% of the cellular ERK2 in immunoprecipitates of Vav.

The tyrosine phosphorylation of Vav was shown to depend on the presence of an active Syk kinase (26). It is possible that Syk might serve as the link between receptor and Vav. Due to the inability to detect small quantities of Syk in Western blots with the available antibody to Syk, we utilized the antibody to immunoprecipitate Syk and probed for the presence of Vav. Fig. 6E shows that in immunoprecipitates of Syk one can detect the presence of trace amounts of Vav. We could not, however, detect any receptor regulation of this association, and this association like that of Grb2 and Raf-1 was resistant to stringent wash conditions.
may occur by phosphorylation-dependent and -independent mechanisms. Nevertheless, aggregation of the receptor increased the association of Vav with receptor, and in immunoprecipitates of Grb2 and Vav from the activated cells, the ability to detect FcεRI chain was greatly enhanced by the aggregation of receptor. What molecule(s) might be responsible for the association of Vav with receptor is unknown although one potential candidate, the tyrosine kinase Syk which associates with the FcεRI chain (10), was also found to co-immunoprecipitate trace amounts of Vav (this study), and its activity was previously shown to be important to Vav phosphorylation (26). This would be consistent with the published reports of the association of the Syk-related kinase ZAP 70 with Vav (19).

Relative quantitation of Grb2, Raf-1, and ERK2 found in association with Vav suggests that between 1 and 3% of the total cellular protein could be found complexed with Vav. While the fraction of molecules in association with Vav is low, these percentages are consistent with other documented protein-protein interactions in vivo (i.e. Lyn or PKC-θ with FcεRI, Fig. 3B) (11, 37). However, we do not exclude the possibility that our solubilization or wash conditions dissociate protein interactions and that in vivo associations are greater. Since Grb2 was previously demonstrated to associate stably with Vav via a dimerization of the SH3 domains of these proteins (20), our findings support a model in which the association of both Grb2 and Vav with the receptor is a consequence of the ability of these molecules to interact with each other. Furthermore, the identical kinetics of Vav or Grb2 association with receptor suggests that these proteins associate as part of a complex (data not shown). This promotes a model of a Vav-associated multi-component complex as a receptor-associated signaling complex (Fig. 8).

The observation that almost 3% of the cellular Raf-1 is also found in association with Vav was somewhat unexpected. This association might suggest an alternative mechanism for membrane targeting of Vav via the association of Raf-1 with Ras (38) or with another unidentified GTP-binding protein. This complex formation may be a prerequisite for receptor association, although Ras was not found complexed to Vav (Table I). Also, proteins that regulate Ras activity (Sos and Ras-GAP) could not be found in Vav immunoprecipitates from activated cells (Table I), and affinity-purified Vav failed to cause guanine
nucleotide exchange from Ras$^2$ in contrast to the activity described in T- and B-cells (15, 16). Experiments in which Vav was depleted from cell lysates of activated cells also did not result in the depletion of Ras-GEF activity. While the presence was depleted from cell lysates of activated cells also did not described in T- and B-cells (15, 16). Experiments in which Vav phospholipase A2 activity (34). The presence of an active Raf-1 nucleotide exchange from Ras2 in contrast to the activity de-

Our results serve to intimately link Vav to the MAP kinase pathway in vivo, in particular to the ERK2 isoform which was shown to be the predominant isoform activated in response to aggregation of the FcεRI (40, 41) and required for cytosolic phospholipase A2 activity (34). The presence of an active Raf-1 in association with Vav shows that a component of the MAP kinase pathway is present in this complex in an active form. Furthermore, our preliminary experiments suggest that Vav-associated ERK2 is also active, and a recent study shows the presence of a small fraction (4% of total MAP kinase) of micro-tubule-associated MAP kinase that is constitutively active in PC12 cells (42). Recently the Vav-dependent induction of nuclear factor of activated T cell activity has been shown to require a functional Ras and Raf, although it is not mimicked by Ras activation alone (43). Our findings do not rule out a role for Raf in activation of Raf-1 in mast cells, but rather suggest that Raf-1 may be activated, or is constitutively active, independently of the activation of the Ras pathway. It is possible that synergy of Ras-dependent and -independent pathways is required for a persistent MAP kinase activation, whereas the individual pathways are capable of only transient activation which may influence differentially cell signaling decisions and responses (44).

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