To study the role of the focal adhesion tyrosine kinase (FAK) in receptor-mediated secretion, we transfected FAK cDNA into a variant (3B6) of the RBL-2H3 mast cell line. This 3B6 cell line expressed low levels of FAK and was defective in high affinity IgE receptor (FceRI) but not Ca\(_{2+}\) ionophore-mediated secretion. FcεRI-mediated secretion was reconstituted after transfection of wild-type FAK. Histamine release was also enhanced by the stable expression of two mutants of FAK: a kinase-inactive form in which the ATP binding site Lys-454 was replaced by Arg or a mutant in which the autophosphorylation site Tyr-397 was replaced by Phe. Therefore, the catalytic activity and the autophosphorylation site of FAK are not essential for secretion. FcεRI aggregation increased the tyrosine phosphorylation of both mutants of FAK to the same extent as wild-type FAK. Therefore, tyrosine kinases activated by FcεRI aggregation are phosphorylating FAK and some of these phosphorylation sites are other than Tyr-397. These results strongly suggest that FAK plays a role in FcεRI-induced secretion by functioning as an adapter or linker molecule.

The focal adhesion kinase (FAK)\(^1\) is a widely expressed tyrosine kinase that localizes with integrins and several proteins to intracellular sites where cells are in contact with the extracellular matrix (1, 2). FAK also becomes tyrosine-phosphorylated in response to the activation of various receptors including the high affinity IgE receptor (FcεRI) and Ag receptors on T-cells (1–4). FAK is essential for development as the inactivation by homologous recombination of the FAK gene results in early embryonic mesodermal defects, retarded growth, and prenatal death (5).

Previously, we reported that the aggregation of FcεRI results in tyrosine phosphorylation of FAK in rat basophilic leukemia RBL-2H3 cells, a mast cell line that has been extensively used to study FcεRI-mediated secretion (3). This receptor-induced phosphorylation of FAK and secretion from these cells are enhanced by adherence of the cells to fibronectin (3, 6). Furthermore, a monoclonal antibody to a ganglioside present on the RBL-2H3 cells induces many of the same intracellular reactions initiated by FcεRI aggregation but fails to result in tyrosine phosphorylation of FAK and does not result in secretion (7). These experiments suggest that FAK may play a role in secretion from mast cells and basophils.

To further define the role of FAK in secretion, we transfected FAK cDNA into a variant of the RBL-2H3 mast cell line that expressed low levels of this kinase and had decreased FceRI, but not Ca\(_{2+}\) ionophore-mediated secretion. FcεRI-induced histamine release was reconstituted in the cell lines that expressed increased levels of FAK. These studies demonstrate a function for FAK in FcεRI-mediated secretion.

**EXPERIMENTAL PROCEDURES**

**Vectors and cDNAs—** Full-length mouse Fak cDNA was generously provided by Dr. S. Hanks (Vanderbilt University, Nashville, TN). pGEM-7Z(-) was from Promega (Madison, WI) and pbLuescript was purchased from Stratagene (La Jolla, CA). pSVL was obtained from Pharmacia Biotech Inc. pSV2-hph containing the hygromycin resistance gene was from ATCC (Rockville, MD).

**Cells and Cell Culture—** RBL-2H3 cells have been characterized previously (8). The 3B6 cells were identified during an attempt to express FAK cDNA into a variant of the RBL-2H3 cells a chimera consisting of the extracellular and transmembrane portions of the interleukin-2a receptor fused to the intracellular portion of human \(\beta\) integrin (the plasmid CMV-IL2R described in (9) is kindly provided by Dr. K. Yamada, NIDR, National Institutes of Health). Expression of this chimeric protein could not be detected by either fluorescence-activated cell sorter or by immunoblotting in cloned cell lines that were resistant to G418. However, we identified two cell lines, 3B6 and 1B6, that had decreased levels of FAK. Morphologically the 3B6 cells are different from the 1B6 in that they contain what appears to be large inclusion bodies or granules. For the present studies, we used the 3B6 cell line, the one that was defective in FcεRI-mediated secretion. The 3B6 cells were cultured in medium containing 100 \(\mu\)g/ml (active concentration) of G418 (Geneticin).

**Site-Directed Mutagenesis—** Point mutations in FAK were generated using the Mutan-Gen Phagmid in vitro Mutagenesis Kit as described by the manufacturer (Bio-Rad, Hercules, CA). For point mutation of Tyr-397 to Phe (Y397F), the C1al-EcoRI fragment of FAK was subcloned in pGEM-7Z(-). The single strand DNA was mutated using the oligonucleotide (5\'-TCTCTGCAAAGTCATC-3\'). To mutate Lys-454 to Arg (K454R), the Sph-I fragment (Y397F) was cloned in pGEM-7Z(-), and the single strand DNA was mutated using the oligonucleotide (5\'-ACAT-GTTCTGATTGCA-3\'). Mutagenized fragments were sequenced to confirm the mutations before subcloning into full-length FAK cDNA. Full-length FAK containing these mutations was subcloned into pSVL at the XbaI-BamHI site for use in transfection studies.

**Transfection Studies—** pSV2-hph containing the hygromycin resistance gene was used for co-transfection. The cDNAs for wild-type or mutated FAK and for pSV2-hph were linearized, and the expression vector and the drug-resistant plasmid at a ratio of 10:1 (55 µg total) were incubated with 3B6 cells for 10 min on ice. After electroporation (Gene Pulser, Bio-Rad, Hercules, CA) at 960 microfarad, 310 mV in Dulbecco’s minimum essential medium, cells were kept on ice for 10 min, then equal aliquots (2 \(\times\) 10\(^6\) cells) transferred to three 100-mm diameter tissue culture plates (Becton Dickinson and Co., Lincoln Park, NJ). Selection (2 mg/ml hygromycin) was started after 48 h. Clones were identified, isolated using cloning rings and expanded for testing.

**Immunoblotting and Immunoprecipitation—** For immunoblotting of whole cell lysates, cells (10\(^6\) cells/well) were washed 2 times with phosphate-buffered saline and immediately lysed with hot sample buffer (75 mM Tris-Cl, pH 6.8; 2% SDS, 10% glycerol). The lysates were boiled for 30 min, and the proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted with the indicated antibodies. For immunoprecipitation of FAK, 2 \(\times\) 10\(^6\) cells were lysed with 1% hot SDS in 150 mM NaCl and 10 mM Tris, pH 7.4. After boiling for 30 min, the proteins were incubated with specific antibodies (anti-FAK, 1:100 dilution) for 1 h at 4 °C. The immunoprecipitates were washed three times with lysis buffer and were used for Western blotting.
Identification of a Cell Line Expressing Low Levels of FAK—During studies on the role of FAK in secretion in RBL-2H3 cells, a variant cell line, 3B6, was identified that expressed low levels of FAK. By immunoblot analysis, the 3B6 cells expressed 16 ± 9% (mean ± S.D., n = 3) as much FAK as RBL-2H3 cells (data not shown). The 3B6 cells were also defective in FceRI-dependent secretion (Fig. 1). FceRI aggregation in the 3B6 cells induced a maximum histamine release of 6 ± 5% compared with 55 ± 10% (mean ± S.D., n = 7) from the RBL-2H3 cells. However, the Ca^{2+} ionophore-mediated histamine release was similar in the 3B6 and RBL-2H3 cells, indicating that the 3B6 cells had a normal distal degranulation pathway. By flow cytometry, the RBL-2H3 and 3B6 cells expressed similar numbers of FceRI (data not shown). The expression levels of the key signaling tyrosine kinases Lyn and Syk were also not decreased in the 3B6 cells (see below). The 3B6 cell line was stable in culture and maintained the phenotype of low FAK and decreased signaling tyrosine kinases Lyn and Syk were also not decreased in the 3B6 cells (data not shown). For immunoprecipitation with other antibodies, lysates from 2 × 10^6 cells prepared with ice-cold Triton X-100 lysis buffer (1% Triton X-100, pH 7.4, 50 mM NaCl, 50 mM NaF, 1 mM Na_3VO_4, 0.5 unit/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride) were incubated with antibodies directly coupled to Sepharose 4B beads. For all immunoprecipitations, the lysates were incubated with the beads for 2 h at 4°C. After washing, the beads were resuspended in SDS-PAGE sample buffer and boiled for 5 min. The proteins in the eluates were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with the indicated antibodies.

Isolation of Cell Lines with Increased Expression of FAK—After FAK cDNA transfection into the 3B6 cells, 16 cloned cell lines were obtained. By immunoblotting of whole cell lysates, five of these cloned lines had levels of FAK that were greater than the parental 3B6; two of these cell lines were excluded from further analysis because in one the expression of FAK was unstable and decreased in culture, and the other cell line grew very slowly (the three cell lines used here are shown in Fig. 2). In contrast, the level of FAK in the remaining 11 lines was similar to the parental 3B6 cells (data not shown). An additional 21 cloned lines that expressed high levels of FAK were isolated by cloning of one of the batch cultures from which the original 16 clones were derived. In the FAK transfected cell lines, the levels of the key signaling molecules Lyn and Syk and the receptor subunit FceRIβ were similar to the parental 3B6 cells (Fig. 2). Flow cytometric analysis also indicated that the parental 3B6 cells and the FAK-transfectants expressed similar levels of FceRI (data not shown). Therefore, by transfection, stable cell lines were isolated that had increased expression of FAK.

FceRI Aggregation Induces Tyrosine Phosphorylation of FAK in the Transfected Cell Lines—We previously reported that FceRI aggregation in RBL-2H3 cells increases tyrosine phosphorylation of FAK (3, 10, 11). Therefore, we examined whether aggregating FceRI would also induce the tyrosine phosphorylation of transfected FAK (Fig. 3). FceRI aggregation in the three transfected cell lines increased the tyrosine phosphorylation of FAK, indicating that at least some of the expressed FAK participates in FceRI-initiated signaling.
Increased Expression of FAK Reconstitutes FcεRI-Induced Secretion—As previous reports suggest a role for FAK in secretion, we examined FcεRI-induced histamine release in the cell lines that had increased expression of FAK (3, 7). FcεRI aggregation induced dramatically more histamine release from the FAK-transfected cell lines than from the parental 3B6 cells (Fig. 5A). In time course studies, the enhanced release was apparent within 2 min of FcεRI aggregation and was maximal by 10–15 min (Fig. 5B). There was also similar enhancement of histamine release when FcεRI-bound IgE was aggregated by specific antigen (data not shown). Receptor-mediated histamine release was also enhanced in all the 21 cell lines that expressed high levels of FAK and that were isolated by cloning of one of the batch culture (data not shown). In contrast, FcεRI-induced histamine release was not enhanced in the 11 cloned cell lines isolated after transfection that expressed levels of FAK similar to that in the parental 3B6 cells (data not shown). Similarly, FcεRI-induced histamine release was not enhanced in the 26 cloned lines isolated by recloning the parental 3B6 (data not shown). Therefore, enhanced secretion was directly related to the expression of FAK in transfected cells.

The Effect of Mutations in FAK on FcεRI-induced Secretion—To study the function of FAK in secretion, mutagenesis was used to generate single-site replacements in the FAK molecule. A kinase-inactive FAK was produced by replacing the ATP binding site Lys-454 with an Arg (K454R) (1, 2). A second mutant form was produced by replacing the autoprophorylation binding site Tyr-397 with Phe (Y397F). In fibroblasts, the phosphorylation of this tyrosine creates a binding site for the SH2 domain of Src family tyrosine kinases (17–22). These mutated forms of FAK were transfected into the 3B6 cells, and cloned lines were isolated. Expression level of FAK was at least equal or greater than in RBL-2H3 cells in 12 lines from the kinase-inactive K454R mutation and in 10 lines transfected with the Y397F mutant. The results with two representative cell lines expressing each of these mutants of FAK is shown in Fig. 6. By in vitro kinase reactions, both Y397F and K454R mutants of FAK did not autophosphorylate (Fig. 7). This is in agreement with previous reports that Tyr-397 is the major site phosphorylated in vitro (19, 20). In cells expressing these mutants, the general pattern of cellular protein tyrosine phosphorylations before and after FcεRI aggregation was similar to that in the 3B6 cells (data not shown).

FcεRI-induced secretion was tested in the cell lines that expressed the mutated FAK at levels similar or greater than that in the RBL-2H3 cells. The release in the 12 cell lines transfected with the kinase-inactive (K454R) was 36 ± 13%, and it was 29 ± 9% (mean ± S.D.) in the 10 lines transfected with the autophosphorylation mutation. The FcεRI-induced histamine release from representative cloned cell lines expressing these two different FAK mutations is shown in Fig. 8. Although some of the cell lines transfected with the mutated forms of FAK expressed less of this protein than cells transfected with the wild-type FAK, there was still reconstitution of histamine release. In contrast to these results, histamine release was not enhanced in two cloned cell lines isolated after transfection that had levels of FAK similar to the parental 3B6 cells. These results indicate that neither the catalytic activity nor the autophosphorylation site are essential for FAK function in FcεRI-induced secretion.

The enhancement of histamine release by the expression of the kinase-inactive or the autophosphorylation mutant was surprising, especially since both proteins failed to auto-phosphorylate in vitro (Fig. 7). However, FcεRI aggregation increased the tyrosine phosphorylation of both FAK mutants in vivo (Fig. 9). These results indicate that cross-linking the receptor induces the phosphorylation of FAK on tyrosine residues other than Tyr-397. Furthermore, FcεRI-induced tyrosine phosphorylation of FAK must be due largely to other tyrosine kinases.

**DISCUSSION**

These results indicate that FAK plays an important role in FcεRI-mediated secretion. The reconstitution of degranulation in transfected cell lines was directly related to the increased amount of FAK in these cells. For example, FcεRI-induced
histamine release was defective in over 50 untransfected and transfected cloned cell lines derived from 3B6 cells that expressed low levels of FAK. However, no direct correlation could be made between the level of wild-type FAK expression and the extent of enhancement in histamine release. For example, although there was less FAK in the transfected 3E6 than in the 2C1 and 2B4 cells (Fig. 2), histamine release was similar in all three lines and was no different than in RBL-2H3 cells (Figs. 1 and 5). However, in these three transfected lines, the amount of FAK was much greater than in the RBL-2H3 cells. Since Fc epsilon RI-induced secretion is the result of a complex cascade of reactions, increasing the level of FAK beyond a certain level may not further enhance histamine release.

Transfection with the kinase-inactive FAK (K454R) indicates that the catalytic activity of FAK is not essential for Fc epsilon RI-mediated secretion. Although K454R failed to autophosphorylate in vitro, Fc epsilon RI aggregation increased the tyrosine phosphorylation of K454R in vitro. This suggests that FAK is phosphorylated by other tyrosine kinases that are activated by Fc epsilon RI aggregation. The phosphorylation of FAK by other tyrosine kinases has also been suggested by other investigators (17, 19–22). Although FAK does not have SH2 or SH3 domains, it interacts with cytoskeletal and other proteins (10, 17, 20, 23–27). Some of these interactions are by the SH2 domains of proteins binding to phosphorylated tyrosine residues in FAK. The tyrosine phosphorylation of the kinase-inactive FAK may allow for such interactions, and therefore, the mutated FAK can still function in transducing Fc epsilon RI-initiated signals.
tyrosine kinases activated by FcεRI aggregation. Therefore, the activation and/or the regulation of these kinases and not the amount of FAK may be the factor that determines the extent of the tyrosine phosphorylation of FAK.

FcεRI-mediated secretion involves complex biochemical reactions including protein tyrosine phosphorylation (31–36). In this pathway, the aggregation of FcεRI results in the phosphorylation on tyrosine residues of the cytoplasmic domain of the β and γ subunits of the receptor. Syk then binds to the tyrosine phosphorylated receptor subunits and is tyrosine phosphorylated and activated to propagate downstream signals including Ca²⁺ influx (14, 15, 37–41). The following data suggest that FAK functions downstream of Ca²⁺ influx. First, Syk is essential for the FcεRI-induced rise in intracellular Ca²⁺ and tyrosine phosphorylation of FAK. Second, FcεRI aggregation does not induce the tyrosine phosphorylation of FAK in the absence of Ca²⁺ in the medium. Third, stimulation of cells with Ca²⁺ ionophore increases intracellular Ca²⁺ and induces the tyrosine phosphorylation of FAK (3).

The signaling pathways initiated by immune receptors on T cells, B-cells and mast cells are similar. Aggregation of these receptors initiates a rapid increase in tyrosine phosphorylation of subunits of the receptors and of several other cellular proteins (35, 36, 42–46). Src family tyrosine kinases are thought to be important for phosphorylating the receptor subunits which then recruits Syk/ZAP70 family tyrosine kinases and leads to the activation of downstream signals. In mast cells, protein tyrosine kinases also mediate late signaling events (47, 48). One such kinase has been identified as FAK (3). This kinase is also present in T and B cells and becomes tyrosine-phosphorylated after immune receptor activation of these cells (3, 4, 49, 50). Interestingly, immune receptors synergize with receptors for adhesion molecules in the tyrosine phosphorylation of FAK (3, 4). Here we show that FAK plays an important role in the signaling pathways leading to secretion in mast cells. Because of the similarities in the signaling pathways of immune receptors, FAK may play a similar role in signal transduction from immune receptors on other cells.

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