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Alterations in Granule Matrix and Cell Surface of Focal Adhesion Kinase-Deficient Mast Cells

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Focal adhesion kinase (FAK) is a nonreceptor protein tyrosine kinase that plays an important role in many cellular processes and is tyrosine phosphorylated after FcεRI aggregation in mast cells. In mice, null mutation of the fak gene results in a lethal phenotype in which the embryos fail to develop past day 8.5 of gestation. To study the role of FAK in these mast cells, 8.5-day embryos were isolated and placed in culture with IL-3 and stem cell factor (SCF). Although FAK was not required for the development of mast cells in culture, the FAK−/− embryo-derived mast cells had several distinct characteristics. Compared with the controls, the mast cells that lack FAK were less metachromatic and by electron microscopy had granules that appeared largely electron lucid, although their histamine content was unchanged. The FAK-deficient mast cells had a reduction in the content of chondroitin/dermatan sulfate, the major glycosaminoglycan component of the granular matrix. The FAK-deficient cells had fewer microvilli that were fused with each other, giving the cell surface a ruffled appearance. There was also a 3-fold increase in the number of cells highly expressing β3 integrin. However, signal transduction from the high affinity IgE receptor for the secretion of histamine was similar in the wild-type, heterozygote, and the FAK-deficient cells. The FcεRI-induced tyrosine phosphorylation of paxillin, Crk-associated tyrosine kinase substrate (CAS), and mitogen-activated protein kinase proteins was independent of FAK. These results indicate that FAK plays a role in regulating the glycosaminoglycan content of the secretory granules and influences the cell surface morphology of mast cells.


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*Receptors and Signal Transduction Section, Oral Infection and Immunity Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, MD 20892; †Departamento de Biologia Celular e Molecular e Bioagentes Patogênicos, Faculdade de Medicina de Ribeirão Preto-Universidade de São Paulo, Ribeirão Preto, Brazil; and ‡ Departamento de Bioquímica, Escola Paulista de Medicina, Universidade Federal do Estado de São Paulo, São Paulo, Brazil

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1 Current address: Center for Cell Biology and Cancer Research, Albany Medical College, Albany, NY 12208.
2 Address correspondence and reprint requests to Dr. Reuben Siraganian, Building 10, Room 1N106, National Institute of Dental Research, National Institutes of Health, Bethesda, MD 20892. E-mail address: rs53x@mail.nih.gov
3 Abbreviations used in this paper: FAK, focal adhesion kinase; CAS, Crk-associated tyrosine kinase substrate; DNP, dinitrophenol; EDMC, embryo-derived mast cell; ERK, extracellular signal-regulated kinase; GAG, glycosaminoglycan; HSA, human serum albumin; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; SCF, stem cell factor; SH, Src homology.
these cells do not demonstrate a defect in FceRI-mediated degranulation. However, our results also show that FAK deficiency results in mast cells with changes in cell surface morphology and in the glycosaminoglycan (GAG) content of the secretory granules. Hence, we propose in this work novel functions for FAK in influencing the structure of the cell surface microvilli and in regulation of the GAG content of mast cell granules.

Materials and Methods

Materials

mAbs to FAK, CAS, and paxillin were purchased from Transduction Laboratories (Lexington, KY), and the HRP-conjugated anti-phosphotyrosine Ab (4G10) was from Upstate Biotechnology (Lake Placid, NY). The tissue culture reagents were purchased from Life Technologies (Rockville, MD). Mouse IL-3 and stem cell factor (SCF) were from Biosource International (Camarillo, CA). mAbs AA4 and the anti-FceRIβ Abs have been described previously (24, 25). Chondroitin 4-sulfate and dextran sulfate were purchased from Miles Laboratories (Elkhart, IN). Heparin sulfate from bovine pancreas was a gift from H. Nader (Universidad Federal Paulista, São Paulo, Brazil). Heparin from bovine intestinal mucosa was obtained from INORP (Buenos Aires, Argentina). Propylenediamine (1,3-diaminopropane) was purchased from Aldrich (Milwaukee, WI); barium acetate and methanol were obtained from Merck, S.A. (Rio De Janeiro, RJ Brazil); agarose standard low-m, was purchased from Bio-Rad (Richmond, CA).

Mice

All animal experiments were conducted with the approval of the institutional Animal Care and Use Committee. Male FAK+/– mice on a C57BL/6 background were kindly provided by D. Ilic (University of California, San Francisco, CA) and were maintained by breeding with C57BL/6 mice and genotyping the progeny (17). For the mast cell culture experiments, the breeding of the mice was performed as follows: 3 females and 1 male FAK+/– mice were placed in a cage and the females were examined every morning for the presence of a vaginal plug. The observation of a vaginal plug was considered day 0.5 of gestation. At 8.5 days of pregnancy, female mice were sacrificed, the uterus was removed, and the pregnancy, female mice were sacrificed on the 5th day of gestation. For the mast cell culture experiments to 5 wk of culture, an aliquot of cells was removed from the mast cell culture supernatants containing nonadherent cells were transferred into 25-cm2 flasks and cultured by adding equal volume of fresh complete medium twice per week. After 4–5 wk of culture, an aliquot of cells was removed from the mast cell culture corresponding to each embryo. The DNA was purified and analyzed by PCR, as previously described (17), to define the genotype of the EDMC, and the expression of FAK was verified by immunoblotting. Equal number of cultures was maintained that had the three genotypes (FAK+/+, FAK+/–, and FAK–/–), and the cells were compared in experiments 5–7 wk after removal from mice.

Cell activation

For histamine release experiments, the cells were cultured for 24 h with anti-trinitrophenol-specific IgE added to the complete medium. The cells were then centrifuged and washed twice with Eagle’s MEM with Earle’s balanced salt solution containing 0.1% BSA and 10 mM Tris, pH 7.4. After the last wash, the cells were adjusted to 4 × 105 cells/ml and stimulated in this medium with several concentrations of the Ag dinitrophenol coupled to agarose standard low-mr was purchased from Bio-Rad (Richmond, CA). Methanol was obtained from InfluChem (INORP (Buenos Aires, Argentina). Propylenediamine (1,3-diaminopropane acetate) buffer, pH 9.0 (28). To distinguish heparin from heparan sulfate and other GAGs, discontinuous electrophoresis was also used. The gel was run for 10 min in 0.04 M barium acetate, pH 5.8, at 5°C (29). The gel was then transferred to another chamber containing dinitrophenol acetate buffer and equilibrated for 15 min at 5°C. The current was then applied in the same direction for 90 min. At the end of the electrophoresis, the gels were fixed with 0.1% cetyltrimethylammonium bromide, dried, and stained with 0.01% toluidine blue in 50% ethanol containing 1% acetic acid, and then destained with the same solution without toluidine blue. The GAGs were quantitated by densitometry at 540 nm using chondroitin sulfate, dermatan sulfate, and heparan sulfate and heparin as standards.

Cell staining

To determine the percentage of metachromatic cells, the cells from each genotype were stained for 15 min in suspension with toluidine blue (4% formaldehyde, 0.1% toluidine blue, and 1% acetic acid, pH 2.8). Aliquots of each genotype were then counted in a hemocytometer, and the percentage of metachromatic cells was determined. Counts were performed in triplicate from five separate experiments. To analyze the intensity of the metachromasia, the cells were fixed for 15 min at room temperature in 4%
formaldehyde, rinsed in PBS, and placed on coverslips coated with Cell Tak (BD Labware, Bedford, MA). Samples were then refixed and stained for 10 min with toluidine blue, dehydrated in a graded series of ethanol and xylen, and mounted in Permount (Fisher Scientific, Pittsburgh, PA). Images were collected using a Nikon E800 microscope (Nikon Instruments, Melville, NY) equipped with a Nikon digital camera model DXM 1200. Intensity of metachromatic staining was quantified using Image-ProPlus version 4.5.1 (MediaCybernetics, Silver Spring, MD). Data were collected from 50 cells from each genotype. This analysis was performed in triplicate from three separate experiments. For berberine sulfate staining, cells were fixed with 4% formaldehyde for 15 min and stained with 0.02% berberine sulfate (Molecular Probes, Eugene, OR), pH 4.0, for 20 min, rinsed in water adjusted to pH 4.0 with acetic acid for 5 min, mounted in anhydrous glycerol, and immediately examined with an Olympus BX-50 fluorescence microscope.

**Immunomicroscopy**

Cells were rinsed in PBS, fixed in suspension in PBS containing 2% formaldehyde (EM Sciences, Fort Washington, PA), rinsed in PBS, placed on Cell Tak-coated coverslips, and permeabilized with methanol for 5 min at −20°C, or for anti-integrin staining the cells were permeabilized in acetic acid for 5 min at −20°C. The cells were rinsed in PBS containing 0.1 M glycine and incubated with mAb AA4 (5 μg/ml) directly conjugated to FITC, mAb BGD6 (20 μg/ml) directly conjugated to FITC, anti-FceRIβ (10 μg/ml), anti a, or anti βc, rat-mouse anti-mouse-anti-rat IgG Alexa 488 (Molecular Probes), or mAbs rat anti-mouse integrin subunits α2, αb, or β2 (20 μg/ml; BD Pharmingen, San Diego, CA) for 1 h at room temperature. Following incubation, the cells were rinsed thoroughly in PBS. The samples incubated with anti-FceRIβ were further incubated with donkey anti-mouse F(ab')2 conjugated to FITC (Jackson ImmunoResearch, West Grove, PA), and the cells incubated with anti-integrin Abs were incubated with donkey anti-rat IgG Alexa 488 (Molecular Probes). All cells were then rinsed and, after the coverslips were mounted with Fluoromount-G (EM Sciences), observed with an Olympus BX-50 fluorescence microscope. The percentage of cells immunolabeled with the various Abs was determined by counting the positive and negative cells from five different fields. A minimum of 100 cells from a minimum of three experiments was counted.

**Electron microscopy**

For transmission electron microscopy, cells were fixed in 2% glutaraldehyde (Ladd Research Industries, Burlington, VT), 2% paraformaldehyde (Ladd) in 0.1 M cacodylate buffer, pH 7.4, containing 0.05% CaCl2, for 40 min at room temperature. Cells were postfixed in 2% OsO4 (EM Sciences) for 1 h at room temperature, rinsed in distilled water, dehydrated through a graded series of ethanol, rinsed in acetone, and embedded in Embed 812 (EM Sciences). Thin sections were cut with a diamond knife and stained for 10 min each in Reynolds’ lead citrate (30) and uranyl acetate. Sections were examined in a Phillips 208 Electron Microscope.

For scanning electron microscopy, cells were fixed, as described above; 100 to 101 cells were plated onto 12-mm round coverslips coated with Cell Tak (BD Labware) and were rinsed twice in 0.1 M cacodylate buffer, pH 7.4, osmicated for 2 h in 1% buffered osmium tetroxide, rinsed in distilled water, and treated for 10 min with 1% aqueous thiocarbohydrazide (EM Sciences). The cells were then osmicated again for 30 min. The last two steps were repeated once. Following this, the cells were dehydrated in ethanol, critically point dried in liquid CO2, and coated with gold. Samples were examined using a JEOL JSM 5200 scanning electron microscope.

**RT-PCR**

Total RNA was extracted from the mouse mast cell cultures using the RNeasy Mini Kit (Qiagen, Valencia, CA). The RNA was treated with DNase I to eliminate residual genomic DNA; cDNA was then synthesized using random hexamer primers. The following pairs of oligonucleotide primers were used for amplification: FceRIα (527 bp), 5'-TGCCCCAGCT GTGCCCTAGAC-3' (sense) and 5'-CCACCTGCTAAGATGCC-3' (antisense); FceRIβ (395 bp), 5'-TGTCCTGTGTGTGCTCTG-3' (sense) and 5'-GTTCCTCCCTCTCTCC-3' (antisense); mouse mast cell protease (395 bp), 5'-CTTCACTTATCCACC-3' (sense) and 5'-CAACACAACACTGAATCG-3' (antisense); mouse mast cell protease 6 (425 bp), 5'-CCATATCCCACCATATCC-3' (sense) and 5'-ACACA CACAGAGAGCCAG-3' (antisense); mouse carboxypeptidase A (689 bp), 5'-ACGAGAGATGCTGGAGG-3' (sense) and 5'-TAATGCAG ACCTCTAGAC-3' (antisense); mouse c-kit (759 bp), 5'-CATCCTCATGCACTACAG-3' (sense) and 5'-AACACTCCGAATCTGCACAGT-3' (antisense); actin (455 bp), 5'-GGCGAAGTCTGTTACTGAGC-3'.

**RESULTS**

**Development of embryo-derived FAK-negative mast cells**

Null mutation of the fak gene results in a lethal phenotype in which embryos fail to develop past day 8.5. Therefore, mast cell cultures were established from embryos at 8.5 days of gestation. FAK+/− mice were crossed, and on day 8.5, embryos were freed of associated uterine tissue with the use of a dissecting microscope, and the cells from each embryo were cultured individually in medium containing both IL-3 and SCF. After ~4 wk of culture, an aliquot of cells was removed from each cell culture and used to define the genotype of the embryos. As expected, 25% of the cultures were FAK+/−, 50% were FAK−/−, and 25% FAK+/+. The genotype of the embryo had no effect on the number of cells obtained in the different cultures, and there was similar proliferation of cells when cultures were starved of growth factors for 12 h and then tested with IL-3 or SCF (data not shown). The expression of FAK was verified by immunoblotting (Fig. 1). Because the level of Pyk2 and paxillin is strongly increased in fibroblasts lacking FAK (17, 20), we also looked at the levels of these two proteins in the cultured mast cells. No difference could be seen between the three genotypes. Similarly, the expression of several other signaling molecules such as Syk was also similar.

The cells that developed in vitro from these embryos had the characteristics of mast cells. By 4 wk, 98−99% of cells in all the cultures had the distinctive surface markers for mast cells: by immunolabeling, the cells all expressed the β subunit of FcεRI (99.6 ± 0.6%), and the rodent mast cell-specific gangliosides (24) recognized by mAb AA4 (98.7 ± 0.5%) and bound IgE (99.3 ± 0.7%). By FACS analysis, the binding of IgE was similar on cells from each of the three genotypes. RT-PCR analysis of the EDMC at 4 and 6 wk of culture showed that the cells contain mRNA for the α and β subunits of FcεRI as well as for c-kit (data not shown).

They also contained mRNA for carboxypeptidase A and the mouse mast cell-specific proteases 5 and 6. Again, the PCR analysis was similar for all three genotypes. The histamine content was also similar between the FAK+/− (335 ± 39 ng/106 cells), the FAK−/− (364 ± 40 ng/106 cells), and the FAK−/+ (352 ± 36 ng/106 cells).
in 12 different mast cell cultures. These data demonstrate that FAK is not required for the development of mast cells in culture.

FAK-deficient EDMC have an altered secretory granule matrix

After 6 wk in culture, the EDMC from all three genotypes (Fig. 2) have a morphology similar to that seen for bone marrow-derived mast cells cultured in IL-3 and SCF (31, 32). The most prominent difference among the genotypes was in the intensity of the metachromatic staining of the cytoplasmic granules with toluidine blue. There was no significant difference in the percentage of metachromatic cells from each genotype, FAK+/+ = 18.4% ± 5.5, FAK+/− = 19.9% ± 3.6, FAK−/− = 21.4% ± 11.8 (data are expressed as percentage of metachromatic cells ± SD), but the intensity of the metachromatic staining of the FAK−/− cells was significantly less than that observed in the FAK+/+ cells. Cells from the FAK+/+ and FAK+/− cultures were similar in appearance, with many cells showing intensely stained cytoplasmic granules (Fig. 2, A and B), but there was a greater heterogeneity in the intensity of the metachromatic staining in the FAK+/− cells. In contrast, the cytoplasmic granules in the cells from the FAK−/− mice were only slightly metachromatic (Fig. 2C). By image analysis (Fig. 2D) of cell cultures from the three genotypes stained with toluidine blue, the intensity of the metachromasia in the FAK−/− cells was 45% less than that of the FAK+/− cells, and the intensity of staining of FAK−/− was 36% less than the FAK+/−.

The difference in the cytoplasmic granules was even more apparent when the cells were examined by transmission electron microscopy (Fig. 3). The majority of the granules in the FAK+/+ and FAK+/− EDMC were electron dense. In contrast, the cytoplasmic granules in the FAK−/− EDMC were largely electron lucid and appeared to have less granule matrix. Therefore, although the mast cells from all three genotypes have equivalent amounts of histamine, the cytoplasmic granules from the FAK−/− cells have reduced levels of metachromasia and their secretory granules were electron lucid.

Because sulfated GAGs are a major component of the mast cell granule matrix and are largely responsible for the metachromasia seen with toluidine blue staining, the content of the GAGs from the three genotypes was analyzed by agarose gel electrophoresis. The EDMC contained a major class of GAGs that had a migration pattern intermediate between chondroitin sulfate and dermatan sulfate (Fig. 4), and a minor amount that migrated in the heparan sulfate/heparin region of the gel. As has been previously reported with bone marrow-derived mast cells cultured with both IL-3 and SCF, there were very little heparin-containing GAGs (31). By scanning densitometry (Fig. 4), the FAK−/− cells contained 33% less chondroitin/dermatan sulfate and twice as much heparan sulfate/heparin as the FAK+/+ EDMC. Using discontinuous agarose gel electrophoresis, the heparan sulfate/heparin band seen in the continuous system now migrates as a slow-migrating heparin or a heparin-like compound, indicating that the cells contain little heparan sulfate (data not shown). The amount of this slow-migrating band was similar in all three cell lines. These results are supported by the virtual lack of staining of the cytoplasmic granules in all the EDMC with berberine sulfate (data not shown). Therefore, there is
a relative decrease in the chondroitin/dermatan sulfate in the FAK−/− cells.

Cell surface of FAK-deficient EDMC is altered

By scanning electron microscopy, there were alterations in the cell surface microvilli on the FAK+/+ and FAK−/− EDMC (Fig. 5). The surface of the FAK+/+ EDMC is covered with thin microvilli (Fig. 5A). In contrast, the microvilli on the FAK−/− cells are somewhat shorter and thicker (Fig. 5B), while the microvilli on the FAK−/− EDMC appear attenuated and fused with each other, giving the cell surface a ruffled appearance (Fig. 5C). The differences in the appearance of the microvilli were also seen by transmission electron microscopy (Fig. 3, A–C).

Integrin subunit β7 is highly expressed in a subpopulation of FAK−/− EDMC

Because of the alterations in the cell surface morphology of the FAK+/+ and the FAK−/− EDMC and the known association between FAK and integrins, we examined the expression of the integrin subunits in these cells. By immunolabeling, the cells of the three genotypes all expressed the αs, α9, αc, β1, and β7 integrin subunits. The expression of the α subunits was similar in EDMC with the three genotypes. However, there was a subpopulation of cells in all three genotypes that expressed the β7 integrin subunits at a higher level (Fig. 6). This subpopulation was increased in the FAK+/+ and the FAK−/− EDMC (the percentage of cells strongly positive for β7 were as follows: FAK−/−, 3.5 ± 1.4%; FAK+/+, 8.6 ± 0.8%; FAK−/−, 11.4 ± 1.1%). Although there were differences in the expression of some of the integrins, the EDMC of all three genotypes adhered equally well to plates coated with fibronectin (data not shown). Therefore, there are subtle changes in the FAK−/− EDMC in the expression of integrins, but the cells still adhere to fibronectin.

FceRI signaling in FAK-deficient EDMC

FAK interacts with several signaling molecules and has been reported to activate several biochemical pathways that include phospholipase Cγ, phosphatidylinositol 3-kinase, and the mitogen-activated protein kinase (MAPK) (3, 33). Similarly, the stimulation of mast cells by FceRI aggregation results in the activation of these same pathways (34). There is also evidence from the rat basophilic leukemia RBL-2H3 cells of a role for FAK during the late stages of FceRI-induced degranulation (21, 22, 35). FAK may also link cell surface receptors to MAPK activation. Therefore, we examined the receptor-induced activation of these pathways in the EDMC.

FIGURE 5. The microvilli on the cell surface of FAK−/− EDMC are altered. The surface of the FAK+/+ EDMC is covered with numerous, thin microvilli (arrows, A), while the surface of the FAK−/− EDMC appears to have fewer, thicker microvilli (arrows, B). The FAK−/− EDMC have a few short microvilli (arrows, C) that appear to have fused.
The present study shows that: 1) FAK is not essential for the development of mast cells in culture; 2) mast cells that develop from FAK<sup>-/-</sup> embryos are less metachromatic due to a decrease in chondroitin/dermatan sulfate in the granules; 3) these cells have altered microvilli on their cell surface; 4) Fc<subÆ</sub>RI-induced degranulation and tyrosine phosphorylation of CAS, paxillin, and MAPK proteins are independent of FAK. These results suggest that FAK plays a role in regulating how embryonic precursor cells develop into mature mast cells.

Role of FAK in mast cell development

Previous studies have suggested that FAK is not essential for the in vitro differentiation of ES cells into mast cells (38). In our study, we report the development of mast cells in vitro from cells of day 8.5 FAK<sup>-/-</sup> embryos, indicating that mast cell precursors are still present in the mutant embryos and not functionally altered even in the presence of the abnormalities affecting the development of the mesoderm (17, 18). Similar numbers of mast cells were obtained from the cultures of wild-type, heterozygote, and FAK<sup>-/-</sup> embryos, and there were no differences in the rate of cell proliferation with IL-3 or SCF (unpublished observations). As would be expected from these observations, the activation by c-<subÆ</sub>kit ligand of Akt and MAPK was similar in the FAK<sup>-/-</sup> and wild-type cells.

The characterization of the cells indicated that the absence of FAK does not block the development of precursors into mast cells. Indeed, the EDMC from all three genotypes were morphologically

**FIGURE 7.** The Fc<subÆ</sub>RI-induced activation of ERK, P38, JNK, and Akt does not require FAK. Wild-type, heterozygote, and mutant EDMC were cultured overnight with IgE, washed, and incubated with the Ag DNP-HSA (10 ng/ml) for the indicated times. After stimulation, the cell pellets were boiled in SDS-PAGE sample buffer, and aliquots were analyzed by immunoblotting using antiphosphospecific ERK, P38, and JNK Abs (A). The extent of activation by Ag of the Fc<subÆ</sub>RI-mediated signaling and degranulation are not affected by the loss of FAK in EDMC.

The EDMC with the three genotypes were sensitized with IgE and then stimulated with Ag, and the phosphorylation state of extracellular signal-regulated kinase (ERK), P38, c-Jun N-terminal kinase (JNK), and Akt was examined in cell extracts using phosphospecific Abs (Fig. 7). The extent of activation by Ag of the MAPK and Akt was similar in the wild-type, heterozygote, and FAK-negative EDMC. Furthermore, the c-kit-induced MAPK and Akt activation were not regulated by FAK in cells that had been cultured overnight in medium with only IL-3 (data not shown). To test for secretion, cells were sensitized overnight with Ag-specific IgE, and then incubated for 40 min with different concentrations of Ag. Although at the lower Ag concentrations there was some decrease in histamine release from the FAK-deficient EDMC as compared with the wild-type cells, this was not statistically significant (Fig. 8). These results indicate that Fc<subÆ</sub>RI-mediated signaling and degranulation are not affected by the loss of FAK in EDMC.

**FIGURE 8.** Fc<subÆ</sub>RI-induced histamine release is independent of FAK. Wild-type, heterozygote, and mutant EDMC were cultured overnight with Ag-specific IgE, washed, and stimulated with the indicated doses of Ag for 40 min. The results are the percentage of the total histamine released in the supernatant after Ag stimulation, and are the mean ± SE of 27 independent experiments per phenotype.

**FIGURE 9.** Fc<subÆ</sub>RI-induced tyrosine phosphorylation of paxillin and CAS

FAK associates with several adaptor proteins, such as paxillin and CAS, and has been suggested to be involved in the tyrosine phosphorylation of these molecules (14–16, 36). Because the aggregation of Fc<subÆ</sub>RI results in the tyrosine phosphorylation of paxillin in mast cells (37), we next examined whether FAK plays a role in this phosphorylation. In preliminary time-course experiments, the maximal tyrosine phosphorylation of paxillin occurred at 5 min after stimulation and decreased to baseline by 10 min (data not shown). The cells were therefore stimulated with Ag for 5 min, and the paxillin was immunoprecipitated and analyzed for tyrosine phosphorylation (Fig. 9A). Aggregation of Fc<subÆ</sub>RI resulted in increased tyrosine phosphorylation of paxillin in the FAK-deficient EDMC, to the same extent as in wild-type EDMC. Similarly, receptor aggregation induced the tyrosine phosphorylation of the adaptor molecule CAS in FAK-deficient cells to the same extent as in the wild-type mast cells (Fig. 9B). Therefore, FAK does not appear to play a role in the Fc<subÆ</sub>RI-induced tyrosine phosphorylation of these adaptor proteins.

**Discussion**

The present study shows that: 1) FAK is not essential for the development of mast cells in culture; 2) mast cells that develop from FAK<sup>-/-</sup> embryos are less metachromatic due to a decrease in chondroitin/dermatan sulfate in the granules; 3) these cells have altered microvilli on their cell surface; 4) Fc<subÆ</sub>RI-induced degranulation and tyrosine phosphorylation of CAS, paxillin, and MAPK proteins are independent of FAK. These results suggest that FAK plays a role in regulating how embryonic precursor cells develop into mature mast cells.
similar to that of cultured bone marrow-derived mast cells. Second, staining with toluidine blue showed that the ratio of metachromatic vs orthochromatic cells was not affected by FAK deficiency, but that the intensity of metachromasia was dramatically reduced in the FAK−/− cells. Third, the mRNA for the mast cell-specific proteases (proteases 5 and 6, and carboxypeptidase A) were also expressed in the mutant cells to the same extent as in the wild-type EDMC.

The FAK−/− cells also displayed alterations in their cell surface microvilli. These modifications are most likely a reflection of an underlying rearrangement of the actin cytoskeleton in the FAK−/− cells. Fibroblasts derived from FAK−/− embryos are round in shape and exhibit a cortical ring of actin instead of the actin stress fibers seen in the wild-type cells (39). A similar disruption of the actin cytoskeleton was observed when primary cultures of chick embryos were treated with antisense oligonucleotides to FAK to reduce the expression of FAK in these cells. These cells that had decreased expression of FAK are rounded with bundles of actin at the cell periphery (40).

Although mast cells developed from the FAK−/− embryos, there were changes in their granules. Indeed, by toluidine blue staining, these cells were only slightly metachromatic. In agreement, electron microscopy indicated granules that were largely electron lucid and appeared to have less granule matrix. The metachromatic staining of the mast cell granules is due to the presence of GAGs in the granule matrix. The composition of the granules depends on the cellular microenvironment (41). In the present experiments, the predominant GAGs in the EDMC that developed in culture with IL-3 and SCF were chondroitin/dermatan sulfate. Sulfated proteoglycans play an important role as the granular matrix for the storage of secretory proteins in mast cells and other exocrine cells (42, 43). For example, there are defects in the mast cells from mice that cannot synthesize heparin due to a mutation in an enzyme that is essential for the biosynthesis of heparin (44, 45). The peritoneal mast cells from these mice fail to express several mast cell protease proteins, and by electron microscopy have lucid areas similar to those seen in the FAK-deficient cells. The changes in GAGs in the FAK-deficient cells were not associated with a decrease in the histamine content of the cells; in contrast, there seem to be greater effects on histamine storage in the heparin-deficient mast cells. Therefore, the changes in the GAGs can explain the morphological differences in the cytoplasmic granules of the FAK-deficient mast cells.

Integrins mediate cell adhesion and cell migration, and regulate gene expression and cell survival. FAK is known to associate with integrins and plays a role in the signal transduction from these receptors. However, there were no detectable differences in the adherence of the FAK−/− compared with the wild-type EDMC to fibronectin-coated surfaces. The loss of FAK increased the fraction of mast cells that highly expressed β1 integrin. This integrin plays a role in the small intestinal mast cell response to parasitic infections. Indeed, there are a significant delay and reduction in the magnitude of mastocytosis together with an impaired host protection in the β1 integrin knockout mice challenged with Trichinella spiralis (46). In agreement, α1β1 integrin is required for tissue-specific homing of intestinal mast cell progenitors (47). Hence, by regulating the surface expression of β1 integrin in mast cells, FAK might play a role in the regulation of intestinal host defense.

**Signal transduction in FAK-deficient mast cells**

Tyrosine phosphorylation and activation of FAK occur after the stimulation of cells by adherence or by the binding of ligands to cell surface receptors (9). For example, FAK links integrin receptors to intracellular signaling molecules that can promote the activation of downstream targets such as the ERK2/MAPK. In FAK-deficient fibroblasts, Pyk2 is overexpressed and compensates for the loss of FAK in signaling ERK2 activation (20). Although the expression of Pyk2 was not increased in the FAK-deficient mast cells, c-kit ligand and FcεRI stimulation still induced ERK2 phosphorylation to the same extent level as in the wild-type mast cells. Similarly, the FcεRI-induced activation of the p38 and JNK in the mast cells was independent of FAK. These results also indicate that in mast cells the activation of the MAPK, and probably downstream events, such as the synthesis of cytokines is independent of FAK.

The signal transduction from the FcεRI is mediated by proteins that include the protein tyrosine kinases Lyn, Syk, Fyn, and Btk; the enzymes, phospholipase C-γ and phosphatidylinositol 3-kinase; adaptor molecules such as SLP-76, Vav, and LAT; and small GTP-binding proteins such as Ras, Rheb, and Rac. Although studies in several systems have documented functional interactions of several of these signaling molecules with FAK, the present results indicate that FAK is not essential for degranulation in mast cells. The histamine release from the cells with the different genotypes was similar at Ag concentrations from 1 to 1000 ng/ml, which induced submaximal and maximal stimulation of the cells for degranulation. There was variation among mast cell cultures from different FAK−/− embryos in the extent of histamine release. In cultures derived from 27 different FAK−/− embryos, the maximal Ag-induced histamine release was ≈20% in 9 different mast cell preparations, whereas only 2 of 27 cultures of the wild-type or the heterozygote mast cells were ≈20%. This suggests that the loss of FAK might decrease the development of some FAK-deficient precursors into mast cells. Although there was less variation in release when the wild-type, FAK−/−, and FAK+/− cells were derived from embryos from the same mother, there still was no consistent difference in degranulation between the different cells. In all these experiments, the stimulation of the EDMC was with suboptimal and optimal concentrations of Ag that activate the cells for release. This Ag/IgE results in a strong response, and it is possible that other combinations of stimuli that result in weaker stimulation may have shown a role for FAK in this response.

In previous experiments, a variant of the rat basophilic leukemia cell line was identified expressing low levels of FAK that had decreased FcεRI, but not ionophore-mediated secretion (35). The stable transfection of FAK markedly enhances the FcεRI-mediated secretion; this does not require the catalytic activity of the kinase, suggesting that FAK is functioning as an adaptor or linker molecule (22, 35). This variant of the RBL-2H3 cells expressing low levels of FAK has a phenotype similar to some of the cultured EDMC that had decreased FcεRI-mediated degranulation and could reflect cells that are transformed without some of the compensatory pathways present in the normally responsive cells. Therefore, other molecules or pathways that are present at higher levels in these embryo-derived mast cells are probably lacking in the RBL-2H3 variant cell line that has decreased FAK expression. Another possibility is that FAK may enhance degranulation only when cells are attached; the RBL-2H3 cells grow as an attached cell line unlike the BMMC that are in suspension.

In FAK−/− fibroblasts there is still tyrosine phosphorylation of proteins known to associate with FAK such as paxillin and CAS (17, 48, 49). Similarly, there were no detectable changes in the extent of the tyrosine phosphorylation of these molecules in the FAK−/− mast cells.

Paxillin is a cytoplasmic protein that binds to FAK and has been suggested to be a possible substrate for this kinase (16, 50, 51). Paxillin is present in focal adhesions as a complex with integrin and FAK. In fibroblasts, this association of paxillin and FAK can
be detected in cells attached to the extracellular matrix as well as in cells in suspension, suggesting that this interaction is independent of adherence to fibronectin (15). Furthermore, paxillin is also overexpressed in these FAK−/− fibroblasts (17), although that was not the case in these FAK-deficient mast cells. FceRI aggregation results in prominent cytoskeletal changes and tyrosine phosphorylation of FAK and paxillin (21, 37). The present experiments, however, indicate that FceRI-induced tyrosine phosphorylation of paxillin does not require FAK. These results are similar to those observed in FAK−/− fibroblasts in which adherence to fibronectin still induced tyrosine phosphorylation of paxillin (17).

CAS is another adaptor molecule that interacts with FAK and is involved in cell migration and proliferation (5, 52–54). The SH3 domain of CAS binds to the proline-rich region of FAK, and there is evidence to suggest that FAK may play a role in the tyrosine phosphorylation of CAS (55–57). However, we found that FAK is not involved in the IgE-induced tyrosine phosphorylation of CAS. In Swiss 3T3 cells, an inhibitor of Rho-associated kinases blocks bombesin-induced FAK and paxillin tyrosine phosphorylation, but not CAS phosphorylation (58). In contrast, the integrin-induced tyrosine phosphorylation of CAS was not affected in FAK-deficient fibroblasts (48). Because tyrosine phosphorylation of CAS is absent in cells lacking Src family kinases (23, 49), these findings imply that CAS tyrosine phosphorylation can occur via FAK-independent signaling pathways that involve Src.

Therefore, although FAK interacts with important signaling molecules, several of which are tyrosine phosphorylated after FceRI aggregation, the signal transduction from this receptor and the tyrosine phosphorylation of several proteins were not changed in FAK−/− mast cells. The FAK−/− mast cells also had defects in their granules and the content of GAGs. These results probably are due to changes in intracellular signaling due to FAK deficiency. FAK associates with integrins, growth factor receptors, and many other signaling molecules. The absence of FAK, although not essential for the development of mast cells, by having an effect on these signaling pathways, regulates the formation of the granular GAGs.

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