Phosphatidylinositol 3-Kinase and Ca\(^{2+}\) Influx Dependence for Ligand-stimulated Internalization of the c-Kit Receptor*

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We have evaluated the role of phosphatidylinositol 3-kinase (PI3-kinase) and Ca\(^{2+}\) influx in ligand-stimulated internalization of the c-Kit receptor. The wild type (wt) c-Kit receptor and YF719, a mutant receptor in which the SH2-mediated binding site for the p85 subunit of PI3-kinase is disrupted, were expressed in DA-1 cells. YF719 internalized with similar kinetics as wt c-Kit although the receptor remained localized close to the plasma membrane. However, in the absence of extracellular Ca\(^{2+}\), or in the presence of the competitive Ca\(^{2+}\) influx blocker Ni\(^{2+}\), the YF719 mutant failed to internalize. Failure to internalize in the absence of Ca\(^{2+}\) was also observed for the wt c-Kit receptor in cells that were pretreated with the PI3-kinase inhibitor, wortmannin. Following stimulation with ligand, clathrin heavy chains were found to co-immunoprecipitate with c-Kit. However, under conditions in which PI3-kinase activity is inhibited and Ca\(^{2+}\) influx is blocked, clathrin failed to co-immunoprecipitate with c-Kit. Our results demonstrate that both Ca\(^{2+}\) influx and PI3-kinase activity influence c-Kit endocytosis, and inhibition of these two signals disrupts the earliest stages of ligand-mediated internalization.

c-Kit is a receptor tyrosine kinase and a member of the subfamily that includes the PDGF, 1 CSF-1, and flt-3/flk-2 receptors. Together with its ligand steel factor (SLF), c-Kit is a key controlling receptor for a number of cell types including hematopoietic stem cells, mast cells, melanocytes, and germ cells. c-Kit is the gene product of the W locus in mice (1), and its ligand SLF is the product of the Sl locus (2, 3). Mutations in either locus severely affect the growth and survival of stem cells of these tissues.

As with other receptor tyrosine kinases, stimulation of the c-Kit receptor with SLF results in the recruitment and tyrosine phosphorylation of SH2-containing second messenger-generating enzymes (4) such as phospholipase C-γ and PI3-kinase (5, 6). The phosphorylated lipid products of these enzymes stimulate a variety of intracellular processes including Ca\(^{2+}\) mobilization and actin reorganization (7–9). Coincident with second messenger generation, the process of receptor internalization is also initiated. Within minutes following ligand binding, receptors cluster in dimers or oligomers and internalize by endocytosis, likely through clathrin-coated pits (10–12). Eventually, clathrin coats are removed, and the remaining vesicles fuse with endosomes, late endosomes, and ultimately lysosomes, resulting in receptor degradation.

Numerous deletion and mutagenesis studies have been carried out to map regions of receptor tyrosine kinases required for ligand-stimulated internalization (13–22). Tyrosine kinase activity (23, 24), autophosphorylation sites (17, 18), and interactions with second messenger-generating enzymes have been implicated. In particular, recruitment and activation of PI3-kinase have been associated with ligand-stimulated internalization of the PDGF receptor (14). Receptors carrying mutations within the cytoplasmic domain that disrupt the PI3-kinase-binding site are impaired in the later stages of endocytosis (25). However, conflicting results with a deletion mutant encompassing this binding site demonstrate no impairment in any stage of endocytosis (16).

Ligand-stimulated endocytosis of c-Kit was investigated by Yee et al. (26). They reported that a c-Kit mutant with no kinase activity was impaired for ligand-stimulated internalization. Individual mutations converting tyrosines 719 and 821 to phenylalanines, however, failed to affect internalization, although the receptor remained localized near the inner aspect of the plasma membrane. However, when both PI3-kinase and Ca\(^{2+}\) influx are inhibited, clathrin fails to co-immunoprecipitate with c-Kit, and receptor internalization is completely prevented. These results show that concurrent inhibition of PI3-kinase activity and Ca\(^{2+}\) influx disrupts the earliest stages of c-Kit internalization.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—gp + e NIH 3T3 packaging cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc., Burlington ON) supplemented with 10% fetal bovine serum (FBS) and antibiotics. DA-1 cells are an interleukin-3-dependent, c-kit-negative murine lymphoma cell line (28). They were routinely grown in RPMI supplemented with 10% FBS, 10-5 μM β-mercaptoethanol, and 2% supernatant from WEHI-3 cells as a source of interleukin-3. Bone marrow-derived mast cells (BMMC) were isolated and cultured as described (29).

The cDNAs for wild type (wt) murine c-kit or mutants in which tyrosine 719 was replaced with phenylalanine were cloned into the LXSNI retroviral expression vector. These vectors were transferred into the gp + e NIH 3T3 packaging cells by electroporation, and transfect-
tants were selected by growth in 400 μg/ml G418 (Life Technologies, Inc.). DA-1s were co-cultured with a confluent layer of pooled gp + e transfectants for 24 h. The non-adherent DA-1s were removed and cultured for an additional 5–10 days in interleukin-3. c-Kit-positive DA-1 cells were enriched by two rounds of cell sorting (Facstar Plus, Becton Dickinson) with an anti-c-Kit conjugated to biotin and followed with streptavidin- and phycoerythrin-conjugated secondary antibodies (Jackson, Westgrove PA). Stable populations of c-Kit expressing DA-1s were then cloned by limiting dilution.

As reported by others (6), we confirmed that the pBS subunit of PI3-kine-co-immunoprecipitates with c-Kit following stimulation of the receptor with SLF but fails to co-immunoprecipitate with YF719 c-Kit receptors with SLF results in an equivalent mitogenic response (27) (data not shown), indicating that PI3-kinase activation is not essential for growth stimulation in this instance.

Production and Labeling of Recombinant SLF—Recombinant murine SLF was produced in soluble form in lent mitogenic response (27) (data not shown), indicating that PI3-kinase-wt or YF719 c-Kit receptors with SLF results in an equivalent mitogenic response (27) (data not shown), indicating that PI3-kinase activation is not essential for growth stimulation in this instance.

Production and Labeling of Recombinant SLF—Recombinant murine SLF was produced in soluble form in Escherichia coli using the pFL-AG.AT'S isopropyl-1-thio-β-D-galactopyranoside-inducible secretion expression vector (Invitrogen). This vector includes an eight amino acid N-terminal FLAG epitope (InterScience, Markham, ON). E. coli containing the pFLAG.AT'S plasmid was incubated overnight at 37 °C in LB with 100 μg/ml ampicillin. This culture was then diluted 20-fold and grown to an A600 of 0.4–0.5 before being induced with isopropyl-1-thio-β-D-galactopyranoside. The cultures were then incubated overnight at 37 °C before they were centrifuged at 10,000 rpm for 20 min. The bacterial supernatant was passed through a 0.22-micron filter and stored at −80 °C with 1 mM CaCl2 and 100 μM phenylmethylsulfonyl fluoride. FLAG-SLF was purified by passing supernatants over a column of Anti-FLAG M1 mouse monoclonal antibodies covalently attached to agarose gel. The column was first equilibrated with 30 ml PBS + 1 mM CaCl2. Bacterial supernatants were then passed over the M1 column three times. The FLAG-SLF fusion protein binding to the affinity column is Ca2+-dependent; therefore, elution of FLAG-SLF can be achieved by adding EDTA. Six elutions with 1 ml of PBS + 2 mM EDTA were performed. These were collected, concentrated, and checked for purity by silver stain. SLF was also conjugated with biotin (Sigma) as described (30).

Internalization Assay, Flow Cytometry—1 × 106 cells of wt and YF719 DA-1 clones were resuspended in phosphate-buffered saline (Life Technologies, Inc.) + 0.5% FBS (PBS/FBS) and washed twice with the same solution. MgCl2 and CaCl2, when added, were at 0.5 and 1 mM, respectively. Biotin-conjugated SLF (b-SLF) was then added to the cells in the same solution. The cells were washed twice with 1 ml of 20% Protein A slurry before being fixed with 3% paraformaldehyde for 10 min on ice. This fixation procedure stabilized the staining pattern for several hours with little disruption of the cellular architecture. Following fixation, cells were washed three times with PBS/FBS and then incubated with streptavidin-conjugated phycoerythrin (Jackson) for 30 min on ice. Cells were then washed twice with PBS/FBS and analyzed by flow cytometry for expression and internalization of c-Kit. Anti-clathrin monoclonal (Sigma) stain was used to detect dead cells. These were gated out and not included in the analysis.

The internalization of c-Kit was confirmed to be a ligand-dependent process using a FITC-conjugated anti-kit antibody (2BS) (Pharminge, San Diego CA) directed at the extracellular domain of c-Kit. This antibody does not compete with SLF binding to the c-Kit receptor. Cells were either first incubated with (or without) SLF for 1 h at 4 °C, followed by a 10-min incubation at 37 °C, washed, and then labeled with FITC-conjugated anti-kit for 20 min at 4 °C. Cells were then analyzed by flow cytometry. In the absence of ligand, receptor levels did not decrease after incubation at 37 °C. However, addition of ligand caused a progressive loss of staining with the anti-kit antibody (data not shown) indicating that internalization of c-Kit is stimulated by ligand binding.

Internalization Assay, Microscopy—1 × 106 cells of wt or YF719 DA-1 clones were resuspended in PBS + 0.5% FBS (PBS/FBS) and washed twice with the same solution. FITC-conjugated SLF was then added to the cells in PBS/FBS at a dilution of 1:100 to 1:50. Cells were incubated on ice with FITC-SLF for 40–60 min. Cells were then transferred to a 37 °C water bath for a short incubation time and then subsequently placed back on ice. Cells were washed three times with ice-cold PBS/FBS and then fixed with 3% paraformaldehyde for 10 min on ice. Control experiments showed that this fixation procedure is sufficient to prevent further movement of receptor into the cell (not shown). Following fixation, cells were washed three times with PBS/FBS. Cells were then incubated with anti-FITC and anti-Txr antibodies conjugated with Texas Red (TXR) (Molecular Probes, Eugene, OR) for 45 min on ice. Cells were washed three times with PBS/FBS and then resuspended in 5 μl of 90% glycerol containing 1,4-diazobicyclo[2.2.2]-octane (purchased from Aldrich) which significantly reduces photobleaching (31). Cells were allowed to settle for 10 min on the microscope slide before applying a coverslip and nail polish. Incubation with glycerol was sufficient to immobilize the cells, while maintaining their three-dimensional structure relatively undistorted. Cells were observed by fluorescence microscope (Leitz DMR/BE) using a 100 × oil-immersion objective with filters for both FITC and TXR. Photographs were always exposed for 90 s. When cells were photographed using both filters, the Texas-Red image was exposed first since it experienced greater photobleaching.

For experiments investigating the co-localization of the c-Kit receptor and SLF, cells were incubated with b-SLF and a FITC-conjugated anti-c-Kit antibody (2BS, Pharminge) for 60 min at 4 °C either in the presence or absence of extracellular Ca2+. Cells were then incubated at 37 °C for 0, 7.5, or 15 min. Cells were washed with ice-cold PBS/FBS and fixed as described above. Cells were permeabilized with 0.1% saponin in PBS/FBS for 10 min at room temperature. Cells were then washed in glycerol/1,4-diazobicyclo[2.2.2]-octane and plated on microscope slides as described above. Control experiments with a FITC-labeled isotype control antibody (Pharminge) or streptavidin-conjugated TXR alone showed no staining (not shown).

Wortmannin—Wortmannin (purchased from Sigma) was dissolved at a concentration of 20 mM in Me2SO and stored in aliquots at −80 °C. Cells were prepared for immunofluorescence microscopy and flow cytometric analysis as described above with the exception that 100 mM wortmannin was added to the binding buffer minutes before the 37 °C incubation.

Immunoprecipitation and Western Blotting—For c-Kit immunoprecipitations, 2.5 × 106 BMSCs per sample were starved overnight in RPMI + 0.5% FBS and then washed twice in PBS + 0.5% FBS. Cells were then incubated with 500 ng/ml SLF at 37 °C for various time points using the indicated conditions and then immediately washed twice in ice-cold PBS/FBS. Cells were then lysed in lysis buffer containing 1% Triton X-100 (Caledon, Georgetown ON), 50 mM HEPS (pH 7.0), 150 mM NaCl, 10% glycerol, 1.5 mM MgCl2, and 1 mM EGTA with the following inhibitors: 500 μM sodium orthovanadate, 10 μM aprotilin, 10 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml soybean trypsin inhibitor, 10 μM NaF, and 1 mM sodium molybdate (all from Sigma). Lysates were then spun at 10,000 rpm for 20 min, and supernatants were incubated with 50 μl of a 20% Protein A slurry (Pharminge Biotech Inc.) and 5 μl of a polyribon tail protein antibody raised against a glutathione S-transferase kit cytoplasmic tail fusion protein or 5 μl of preimmune serum and 50 μl of a 20% Protein A slurry. A whole cell lysate prepared from 1/10th the number of cells was used as a positive control on the Western blot. All the other lysates were incubated for 2 h at 4 °C, and the beads were washed three times in HNTG wash buffer containing 0.1% Triton-X, 20 mM HEPS (pH 7.0), 10% glycerol, 150 mM NaCl, and 1 mM sodium orthovanadate. Beads were resuspended in loading buffer with β-mercaptoethanol and boiled for 5 min, and released proteins were resolved on a 7.5% acrylamide gel, transferred to nitrocellulose, and blocked in PBS plus 5% skim milk powder and 0.5% Tween 20. Blots were incubated with an anti-clathrin monoclonal antibodies (from Transduction Laboratories, Kentucky) at a dilution of 1:5,000 overnight followed by goat anti-mouse-conjugated horseradish peroxidase secondary antibodies (Jackson) at a dilution of 1:2,000 and visualized with chemiluminescence (NEN Life Science Products). Blots were stripped by acid treatment and re-probed with rabbit polyclonal anti-c-Kit antisera at a dilution of 1:500 followed by incubation with protein A-horseradish peroxidase (Amersham Corp.) at a dilution of 1:30,000. Visualization was again by chemiluminescence.

RESULTS

Fluorescence-activated Cell Sorter Analysis of Internalization—We used labeled ligand in conjunction with flow cytometry to follow the average rate of internalization over a population of cells. Labeled ligand offers the advantage of following
only those receptors that are activated, unlike the use of anti-receptor antibodies that cannot distinguish between activated and unactivated receptors. Cells were incubated with b-SLF at 4 °C and then further incubated for varying amounts of time at 37 °C. Internalization was arrested by adding ice-cold PBS/FBS and shifting the cells back to 4 °C. Following washing and fixing, the cells were then incubated with Streptavidin-conjugated phycoerythrin and analyzed for surface expression. Fig. 1 demonstrates typical flow cytometry profiles with b-SLF, showing a decline in the intensity of the fluorescence signal as the bound ligand is internalized with the c-Kit receptor.

**Internalization Kinetics of Wild Type and YF719 c-Kit Receptors**—Recruitment and activation of PI3-kinase has been implicated in both endocytic and exocytic processes in a number of different experimental systems. We therefore investigated the ligand-stimulated internalization kinetics of both wt and YF719, a c-Kit receptor mutant that fails to recruit and activate PI3-kinase. Fig. 2 depicts the average of three flow cytometry experiments. The mean fluorescence intensity of cells that were maintained at 4 °C is denoted as 100% surface receptor expression. As shown in Fig. 2A, wt c-Kit and the YF719 mutant expressed in DA-1 cells internalize with similar kinetics. The half-life for loss of surface label is approximately 6 min.

**Receptor Internalization Dependence on Ca2+**—Another early signaling event following ligand-stimulated activation of c-Kit is the mobilization of Ca2+ from intracellular stores, followed by Ca2+ influx from the extracellular milieu (32). A number of Ca2+-binding proteins are associated with coated pits and endosomes, suggesting that high levels of intracellular Ca2+ may influence receptor endocytosis (33–35). We therefore investigated the effect on receptor internalization of removing extracellular Ca2+. As shown in Fig. 2B, when Ca2+ is excluded from binding and wash buffers, wt c-Kit internalizes with kinetics similar to that observed in the presence of Ca2++; however, the YF719 receptor fails to internalize.

To confirm that Ca2+ influx is required for YF719 internalization, Ni2+, a competitive blocker of Ca2+ influx channels (36), was added to the incubation medium containing Ca2+, and the internalization kinetics were determined. As shown in Fig. 2C, the inclusion of 2.5 mM Ni2+ in the binding and wash buffers blocks internalization of the YF719 mutant but has little effect on the kinetics or the extent of internalization of wt c-Kit. Taken together, these results demonstrate that internalization of YF719 is dependent on Ca2+ influx.

**Fluorescence Microscopic Analysis of Internalization**—Receptor endocytosis is a multi-step process involving transfer of receptor from clathrin-coated pits, to early and late endosomes (12). PI3-kinase activity has been particularly associated with movement of receptor down later steps in the endocytic pathway (25). We therefore examined the distribution pattern of internalized wt and mutant c-Kit receptors following activation with SLF using fluorescently labeled ligand and fluorescence microscopy. Cells were incubated with FITC-conjugated SLF at 4 °C to allow ligand binding. Cells were then incubated for a further period at 37 °C to allow for internalization. Following washing and fixing, the cells were further stained with anti-FITC antibody coupled with Texas Red. Because cells were not permeabilized, the anti-FITC antibody binds only those occupied receptors remaining on the surface of the cell. As shown in Fig. 3A, when wt cells are incubated with FITC-SLF at 4 °C, or
for only a short time at 37 °C, the FITC and Texas Red stains are superimposable indicating that the ligand-receptor complex is on the cell surface. With longer incubations at 37 °C, the fluorescence becomes increasingly punctate, and there is an accompanying loss of Texas Red staining. Finally, after 10–15 min of incubation at 37 °C, little Texas Red fluorescence is observed, whereas the majority of the FITC staining is in large aggregates and appears to be internal to the cells. This pattern of staining is consistent with a process involving ligand-driven aggregation of the receptor, followed by internalization of the ligand-receptor complex and subsequent movement down the endocytic pathway.

The receptor distribution pattern following ligand binding was also investigated for the YF719 mutant. As shown in Fig. 3B, in the presence of Ca2+, the YF719 receptor was also observed to aggregate and internalize. However, unlike the wt receptor, the distribution pattern of the YF719 receptor remained punctate and was primarily located near the interior of the plasma membrane, even after a 15-min incubation. This suggests that although the YF719 receptor is internalizing, further movement down the endocytic pathway may be impaired.

When Ca2+ was omitted from the binding and wash buffers, the wt receptor internalized and displayed a pattern of staining identical to that observed in the presence of Ca2+ and with similar kinetics (Fig. 4A). In contrast, although the YF719 receptor was still observed to aggregate, the Texas Red and FITC stains remained co-localized on the surface of the cell (Fig. 4B). This observation indicates that the receptor-ligand complexes are not internalizing, in agreement with our flow cytometric data which also demonstrated an inability of the YF719 receptor to internalize in the absence of Ca2+. We have extended our observations to time points as long as 30 min. At these times, the YF719 mutant still fails to internalize in the absence of Ca2+.

**SLF and c-Kit Remain Associated in Intracellular Compartments**—The acidic environment of endosomes and lysosomes may, in some cases, lead to dissociation of ligand and receptor, resulting in ligand degradation and recycling of receptors back to the cell surface (37). Since we used labeled ligand to follow the fate of the SLF-c-Kit complex, it was important to verify that SLF and c-Kit remained associated in intracellular compartments. Wild type and YF719 DA1 cells were incubated with both b-SLF and a FITC-conjugated anti-c-Kit antibody at 4 °C followed by 37 °C incubations. Cells were then fixed, permeabilized, and incubated with streptavidin-conjugated TxR. As shown in Fig. 5, after a 7.5-min incubation at 37 °C, co-localization of the TxR and FITC labels is observed for both the wt and mutant YF719 receptor, either in the presence or absence of extracellular Ca2+. This co-localization is also observed after a 15-min incubation at 37 °C (data not shown). This observation therefore confirms that receptor and ligand

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**Fig. 3. Fluorescence microscopic analysis of wt and YF719 c-Kit internalization.** DA-1 cells expressing wt (A) or YF719 (B) c-Kit were incubated with FITC-SLF for 60 min at 4 °C. Cells were then incubated at 37 °C for 0, 7.5, or 15 min. Cells were then fixed and stained with Texas Red-conjugated anti-FITC antibody before plating on microscope slides and examining under the fluorescence microscope.

**Fig. 4. Fluorescence microscopic analysis of wt and YF719 c-Kit internalization in the absence of extracellular Ca2+.** DA-1 cells expressing wt (A) or YF719 (B) c-Kit were incubated with FITC-SLF for 60 min at 4 °C in the absence of extracellular Ca2+. Cells were then incubated at 37 °C for 0, 7.5, or 15 min. Cells were then fixed and stained with Texas Red-conjugated anti-FITC antibody before plating on microscope slides and examining under the fluorescence microscope.
remain associated during the internalization process.

_Wortmannin Inhibits Wild Type Internalization in the Absence of Ca\textsuperscript{2+}—_Our observation of impaired receptor internalization in the absence of Ca\textsuperscript{2+} for the YF719 mutant cannot distinguish between a dependence on PI3-kinase binding to the c-Kit receptor or a further requirement for PI3-kinase reaction products generated by enzymatic activation. To address this question, we performed our internalization experiments following pretreatment of the cells with wortmannin, a specific inhibitor of the PI3-kinase enzyme (38). We prepared wt DA-1 cells for either flow cytometry or fluorescence microscopy. Minutes before incubation of the cells at 37 °C, wortmannin was added to the binding buffer. In the presence of Ca\textsuperscript{2+}, and following wortmannin pretreatment, the wt c-Kit receptor was observed to internalize (Fig. 6B). However, when analyzed by fluorescence microscopy, the receptor appeared to localize predominantly at the cell membrane, with a punctate staining pattern similar to that observed for the YF719 receptor (Fig. 7). This altered subcellular distribution suggests that PI3-kinase may be particularly important in later endocytic steps. As shown in Fig. 6D, wt c-Kit receptors on the surface of cells incubated with wortmannin are not internalized in the absence of Ca\textsuperscript{2+}. A failure of the wt receptor to internalize in the absence of Ca\textsuperscript{2+} and following wortmannin pretreatment is also observed by fluorescence microscopy (Fig. 7). Titration of the concentration of wortmannin both in the presence and absence of Ca\textsuperscript{2+} revealed that the amount of wortmannin required to achieve 50% inhibition of c-Kit internalization on BMMCs after a 15-min incubation with SLF at 37 °C was approximately 1 nM (Fig. 6E). This level of wortmannin has been shown to be specifically inhibitory for PI3-kinase enzymatic activity in other systems (38, 39). In contrast, there was no inhibitory effect on internalization in the presence of Ca\textsuperscript{2+} at any concentration of wortmannin. These results therefore indicate that there is a dependence on PI3-kinase enzymatic activity and Ca\textsuperscript{2+} in c-Kit receptor internalization.

_c-Kit Receptor Internalization Is Clathrin-associated_—Our results indicate that ligand-stimulated receptor internalization is modulated by PI3-kinase activation and Ca\textsuperscript{2+} influx. One of the earliest steps in endocytosis is association with clathrin-coated pits. However clathrin-independent endocytic mechanisms have also been reported (23). To determine if c-Kit internalization is clathrin-associated, c-Kit immunoprecipitates were analyzed for the co-immunoprecipitation of clathrin.

Cells were starved overnight, washed, and then stimulated at 37 °C with SLF with or without Ca\textsuperscript{2+} and with or without wortmannin. The cells were washed, lysed, and immunoprecipitated with either preimmune serum (Fig. 8, 1st lane) or anti-kit antibodies (3rd to 14th lanes). 2nd lane contains whole cell lysates using 1/10th the amount of cellular material. Samples were separated by SDS-polyacrylamide gel electrophoresis and analyzed by Western blot. The blot in the upper panel in Fig. 8 was probed with an anti-clathrin heavy chain monoclonal antibody. This blot was stripped and re-probed with anti-kit antibodies (bottom panel of Fig. 8). As shown in the 2nd lane, an immunoreactive band is observed in whole cell lysates that migrates at 180 kDa, the expected molecular mass for the clathrin heavy chain. In the absence of stimulation, only very low levels of clathrin co-immunoprecipitated with anti-c-Kit antibodies (Fig. 8, 3rd lane). No clathrin was observed following immunoprecipitation with c-Kit preimmune serum (Fig. 8, 1st lane). As shown in Fig. 8, 5th lane, stimulation with SLF at 37 °C for 5 min increased the level of clathrin that co-immunoprecipitates with c-Kit. Increased co-immunoprecipitation of clathrin was also observed following stimulation of cells with SLF for 2.5 or 5 min in the absence of Ca\textsuperscript{2+} only or in the presence of wortmannin only (6th to 11th lanes). However, in...
**DISCUSSION**

Ligand-stimulated receptor internalization is a multi-step process involving assembly of an endocytic machinery composed of clathrin heavy and light chains, adaptors, dynamin, and other cytosolic factors. In addition to these elements, recruitment and activation of enzymes such as PI3-kinase have also been implicated in the endocytic process. We found that ligand-stimulated internalization of the c-Kit receptor is blocked when both PI3-kinase activation and Ca\(^{2+}\) influx are inhibited. Although inhibition of either of these signals alone did not prevent the early stages of internalization, loss of PI3-kinase activity resulted in internalized receptors that appeared to accumulate in vesicles close to the membrane. This observation is consistent with the results of Joly et al. (25) who demonstrated that a mutant PDGF receptor that cannot associate with PI3-kinase is internalized but not degraded.

PI3-kinase is a second messenger-generating enzyme that has been linked to mitogenesis, receptor trafficking, and maintenance of cell viability in other receptor systems (14, 27, 40). Indirect evidence suggests a role for PI3-kinase in vesicular sorting or movement of proteins in the cell. Vps34, a yeast protein with homology to the p110 catalytic subunit of PI3-kinase, is involved in the transport of soluble hydrolases from the trans Golgi network to yeast vacuoles (41). Kapeller and Cantley (42) have shown that activated PDGF receptor and PI3-kinase remained complexed in endosomes and associate with microtubules in 3T3-L1 cells. Inhibition of PI3-kinase has also been reported to inhibit transferrin receptor endocytosis, nonspecific fluid phase uptake, and early endosome fusion, possibly via the small GTPase Rab5 (43). In addition, PI3-kinase activation is required for the reorganization of actin filaments and the induction of membrane ruffling by PDGF (7). Besmer and colleagues (26) found little to no effect of a YF719 c-Kit mutation on ligand-stimulated internalization. However, the internalization experiments reported by these researchers were performed under conditions permitting Ca\(^{2+}\) influx, which our results show are critical for internalization in the absence of PI3-kinase.

Although clathrin-independent endocytosis has been reported (10), our observation that clathrin co-immunoprecipitates with c-Kit following stimulation with ligand indicates that internalization of this receptor is clathrin-associated. Importantly, inhibition of either PI3-kinase or Ca\(^{2+}\) influx alone did not prevent this association, suggesting that under either of these conditions, c-Kit internalization remains clathrin-associated. We did, however, fail to observe clathrin co-immunoprecipitation with c-Kit when both PI3-kinase and Ca\(^{2+}\) influx were inhibited. This result indicates that the internalization block under these conditions may be occurring at the earliest step of clathrin cage formation.

C-Kit activates phospholipase C-\(\gamma\) (5, 8) resulting in the hydrolysis of phosphatidylinositol 4,5-bisphosphate into diacylglycerol and inositol 1,4,5-trisphosphate (9). Inositol 1,4,5-trisphosphate causes the mobilization of intracellular Ca\(^{2+}\) from stores in the endoplasmic reticulum, resulting in the stimulation of Ca\(^{2+}\) influx from the extracellular milieu through the Ca\(^{2+}\) release activated channel (32). This Ca\(^{2+}\) influx step seems to be critical for c-Kit internalization, since intracellular mobilization should still occur in the absence of extracellular Ca\(^{2+}\) or in the presence of Ni\(^{2+}\).

Our results do not identify the Ca\(^{2+}\)-dependent step in the early stages of receptor endocytosis. However, a number of Ca\(^{2+}\)-dependent elements implicated in endocytosis have been identified. Clathrin light chains are Ca\(^{2+}\)-binding proteins, and high levels of Ca\(^{2+}\) are thought to stabilize the clathrin cage (44, 45). The adaptor protein annexin VI, a Ca\(^{2+}\)-dependent phospholipid-binding protein, is required for budding of clathrin-coated pits in a cell-free system (34). However, annexin VI was not found to have a role in coated pit formation and constriction nor did it enhance transferrin receptor endocytosis in A431 cells (46). Another Ca\(^{2+}\)-dependent protein is calmodulin, a cytoplasmic mediator of many calcium-regulated processes (47). Calmodulin has been demonstrated to bind to clathrin light chains in a Ca\(^{2+}\)-dependent manner (48), has been shown to regulate endosome fusion in vitro (35), and is implicated in other membrane trafficking events (49). Future experiments will be required to more fully determine the role of these elements in the Ca\(^{2+}\)-sensitive phase of c-Kit internalization.
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