Identification of UNC119 as a Novel Activator of SRC-type Tyrosine Kinases*  

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Lyn, an SRC-type tyrosine kinase, is associated with the interleukin (IL)-5 receptor in eosinophils. The mechanism of its activation is unknown. Through yeast two-hybrid screening we have cloned and characterized a new signaling molecule, Unc119, that associates with IL-5Rα and SRC family tyrosine kinases. Unc119 induces the catalytic activity of these kinases through interaction with Src homology 2 and 3 domains. IL-5 stimulation of eosinophils increases Unc119 association with Lyn and induces its catalytic activity. Lyn is important for eosinophil survival. Eosinophils that are transduced with Unc119 have increased Lyn activity and demonstrate prolonged survival in the absence of IL-5. Inhibition of Unc119 down-regulates eosinophil survival. To our knowledge Unc119 is the first receptor-associated activator of SRC family tyrosine kinases.

One of the fundamental goals of cell biology is to understand the mechanism of signal generation by receptors. Many receptors rely upon kinases, especially tyrosine kinases, for receptor phosphorylation and activation of signaling cascades. The SRC family tyrosine kinases (SRC TKs) are frequently serine kinases that serve as the triggers for cytosolic signals (1). Receptor-associated SRC TKs exist in a non-active conformation and become transiently activated following ligand binding and receptor oligomerization (2). How receptor oligomerization leads to the activation of SRC TKs is unknown.

Two intramolecular interactions tightly regulate structural conformation and enzymatic activity of SRC TKs. One is through SRC homology 2 (SH2) and the other is through the SH3 do-

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§ The abbreviations used are: SRC TKs, SRC family tyrosine kinases; IL, interleukin; HRG4, human retinal gene protein 4; SH2, SRC homology 2 domain; SH3, SRC homology 3 domain; GST, glutathione S-transferase; PBS, phosphate-buffered saline; RT, reverse transcribed; PVD, poly-
vinyldene difluoride; FITC, fluorescein isothiocyanate; HSA, human serum albumin; PI3K, phosphatidylinositol 3-kinase.

MATERIALS AND METHODS

Yeast Two-hybrid Screening—A human fetal cDNA library (Clontech) was screened using the LexA Matchmaker Yeast Two-hybrid System (Clontech). The bait was constructed by fusing the cytosolic and transmembrane portion of IL-5Rα in-frame with LexA DNA binding domain in pLexA plasmid. The cytosolic portion of IL-5Rα (98 amino acids) was PCR-amplified from IL-5Rα cDNA (a kind gift from Dr. J. Tavernier) using AGCGAATTC-AGTGAGTGGAGCCAACCTA and AGAGGATCC-GCATGTGCTAGTCATCA as 5′ and 3′ end primers, respectively, and ligated into pLexA after EcoRI and BamHI restriction enzyme (Invitrogen) reactions. The correct reading frame was confirmed by sequencing. The bait construct and the fetal human cDNA library in the pB42AD (Clontech) were cotransformed into EGY48 yeast strain carrying the reporter plasmid pBopLacZ. A set of negative and

receptor-bound SRC TKs are frequently found to be dephosphorylated under basal conditions, yet lack appreciable catalytic activity (5). CD45-negative cells have variable effects on SRC TKs. CD45 negatively regulates Lyn by dephosphorylating both its negative and positive regulatory tyrosine residues (6). The activity of Lyn and Hck is increased in macrophages (7) and B cells (8) lacking CD45.

Regulation of SRC TK activity through other intramolecular interactions was realized after the crystal structures of Src (9) and Hck (10) were solved. The linker region that is located between the SH2 and the kinase domains binds to the SH3 domain of the kinase rendering a non-active conformation. Furthermore, a short amino acid stretch connecting the SH2 and SH3 domains has recently been identified as an additional negative regulator of Hck and SRC activation (11). The SH3 domain of SRC TKs prefers to bind to the RXTXXP motif (a motif is defined as a short stretch of amino acid residues that binds to a signaling domain) (12, 13). Although the linkers of SRC TK bind to the SH3 domain, they do not have an optimal SH3-binding motif. Src and Fyn do not even have the second proline residue in the motif (13). Although Lyn has the canonical RXTPXP motif (amino acids 230–233) like all other SRC TKs, it lacks the flanking arginine residue. This “imperfect” SH3 motif with low affinity binding can be easily displaced by a high affinity SH3 ligand. Accordingly, the exogenous SH3 ligand NeF (a human immunodeficiency virus protein) activates Hck, a member of SRC TKs, in an SH3 motif-dependent manner (14). However, the identity of endogenous SH3 ligands that activate SRC TKs is largely unknown.

Our laboratory has been interested in the signal transduction mechanism of IL-5R, which is composed of a unique α and a common β subunit (15). The earliest event of IL-5R signaling in eosinophils is the activation of SRC TKs and Janus kinases (16–18). One of the SRC TKs that plays a dominant role in eosinophils is Lyn kinase. We and others (19–21) have shown that Lyn is important for eosinophil survival and differentiation. The molecular mechanism of activation of Lyn kinase by IL-5 receptor is unknown.
positive controls was carried out according to the protocol provided. The transformed colonies were grown on SD/-His- Trp- Ura selection plates for 5 days. The colonies were then replica-plated on induction plates SD/Gal-Raf-His-Leu-Trp-Ura plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Invitrogen). The green blue colonies on induction plates were streaked on selection plates and then again on induction plates to allow possible plasmid segregation and eliminate transition interactions. A stock for each of the double positive colonies (Leu+, LacZ+) and green blue colonies was prepared and stored at -80 °C. These colonies then were individually cultivated in SD/-Trp media overnight for plasmid segregation and selection for pB42AD-cDNA constructs. The plasmid DNA was isolated (22) and electroporated (23) into Escherichia coli KC8 cells. The transformants carrying pB42AD-cDNA were selected on SD/-His-Trp-Ura plates for tetracycline resistance (Invitrogen). The plasmids were purified from individual colonies using QiAprep Spin Miniprep kit (Qiagen) and were cut with EcoRI and XhoI restriction enzymes (Invitrogen) for confirmation of cDNA inserts which was then partially sequenced using pB42AD sequencing primers at the University of Texas Medical Branch Molecular Biology Core Lab. The partial sequence of each colony was searched for homology to known sequences with the BLAST program (www.ncbi.nlm.nih.gov/BLAST) with non-redundant data base.

Primers, RT-PCR, and Southern Hybridization—Primers were designed using the Primer Designer software (1990—1991 Scientific and Educational software) and synthesized at the University of Texas Medical Branch Molecular Biology Core Lab. The PCRs were carried out in a thermal cycler (Perkin-Elmer) in 50 μl PCR tubes. For pB42AD-cDNA, RNA was purified from eosinophils using TRizol (Invitrogen). Twenty nanograms of RNA was reverse-transcribed into cDNA using SuperScript II preamplification kit (Invitrogen) following the protocol provided. PCR amplification was performed using CCGGAAGGCGCAT-GAAGGTGA and CATGCTGGATCTCATGGCC as 5’ and 3’ primers, respectively. The PCR mixture was separated on 3% NuSieve 3:1 agarose gel (FMC Bioproducts) and then either transferred to nylon membrane (Schleicher & Schuell) and hybridized with 32P-labeled (Amersham Biosciences) Unc119 labeled (labeled with DECAprime II kit, Ambion) cDNA sequence of Unc119 was PCR-amplified using GGCGAATTC-CATGAAGGTGAAGAGG and AATGTCGA-GTGGGATACGGGTGTT as 5’ and 3’ end primers, respectively. The PCR fragment was fused in-frame into pGEX-4T2 (Amersham Biosciences) after EcoRI and SacI restriction (Invitrogen) reactions. After the correct reading frame was confirmed with sequencing, it was electroporated into the E. coli BL21 strain. The expression and purification of recombinant Unc119 was performed with the modification of the protocol provided. Briefly, the transformed cells were grown in 250 ml YTA medium overnight with 200 rpm shaking at 37 °C. The overnight culture was diluted 1:100 and grown in 2 liters of M9 medium with enhanced chemotaxis (100 ml each of the protease inhibitors aprotinin, leupeptin, and pepstatin, and 1 mM phenylmethylsulfonyl fluoride. After incubation on ice for 30 min, detergent-insoluble materials were removed by centrifugation at 4 °C for 12,000 × g. After preclearing, 1–2 μg of an appropriate antibody was added to the lysate and incubated at 4 °C for 1 h followed by addition of 20 μl of protein A/G-agarose. The incubation was continued for 2 h or overnight in the case of overexpression. The next day, it was centrifuged at 35,000 × g for 5 min, and the pellet was washed with 1 ml of lysis buffer 5 times or 3 times with lysis buffer and 3 times with kinase buffer in the case of kinase reactions. The samples were separated with SDS-PAGE and blotted onto nitrocellulose or PVDF membranes for immunoblotting. The membranes were incubated in 5% non-fat dried milk or 5% bovine serum albumin/TBS-T. The membranes were then washed 3 times in the TBS-T buffer for 10 min each and incubated with horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences) solution (0.01 μg/ml). After washing again the membranes were developed using enhanced chemiluminescence (ECL or ECL Plus) substrate (Amersham Biosciences). To strip and reprobe, the membranes were incubated in the stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) at 55 °C for 30 min, washed, blocked, and immunoblotted with a proper antibody as explained above. Densitometric analyses of select Western-blotted protein bands were performed with the software “Metamorph” version 6.2r8 (Universal Imaging Corporation, Downingtown, PA).

GST Pull-down—GST or GST fusion proteins (Unc119 or Lyn) were incubated with the cell lysates or recombinant Unc119 at 4 °C for 2 h. Twenty μl of glutathione-agarose was then added, and incubation was continued for 2–4 h or overnight. The samples were centrifuged at 12,000 × g for 5 min, and the pellets were washed with lysis buffer, separated with SDS-PAGE, transferred to PVDF membrane, and Western-blotted.

In Vitro Kinase Assay—To assess the effect of Unc119 on the activation of individual kinases, Lyn, Hck, Ick, or p38 was immunoprecipitated with respective antibodies (Santa Cruz Biotechnology) in bulk with anti-GST or anti-Coomassie blue staining. The respect cell lysates or recombinant Unc119 was immunoprecipitated from the lysates corresponding to 50–60 million cells, aliquoted in 20–30 samples, and kept at -80 °C for future kinase assays. The kinase assays were performed in the presence or absence of Unc119 or its SH3 motif (P15SH3, QRKQPQFGPED), proline to alanine mutant (P15A5, QARQAIAGED), SH2 motif (P15SH2, TCEHYDDFFPLS), or decorated SH2 motif (P15SH2*, DQHGGGFFPLS) or a mimic poly- glutamic acid (P15, SERLPLNRRDDLPNAGRC), or an SH3 peptide from CD2 (CD2, P15SH3, QRGPLPPRVPQKVPPPCG) (28) in a kinase buffer containing 20 mM Tris, pH 7.4, 2 mM MgCl2, 0.5 μM cold ATP, and 2 μCi of [γ-32P]ATP for 5 min. In the reactions in which the effect of Unc119.
on the activation of Lyn was evaluated, 2 μM Enolase (Sigma) or ATF-2 (Santa Cruz Biotechnology) was added to the reactions as the substrate. This buffer was modified from Moarefi et al. (14) to decrease the high base-line phosphorylation of Lyn. The reactions were carried out at 30 °C for different times and were stopped by addition of 6X Laemmli’s buffer. The reactions then were separated by SDS-PAGE, transferred to PVDF membrane, and autoradiographed.

**RESULTS**

**Construction of Bait and Cloning Unc119**—In order to search for IL-5Ra-interactive signaling molecules, we have used the LexA Matchmaker Yeast Two-hybrid System. We constructed the bait pLexA-5Rα by fusing the cytoplasmic tail of IL-5Ra to the DNA binding domain of LexA. By using this bait, we have screened the human fetal liver cDNA library for IL-5Ra interacting proteins. One of the cDNAs was HRG4/ human Unc119 (98% homology) (GenBank™ accession number U40998) with a 1.4-kbp cDNA and a 240-amino acid open reading frame (25).

**Unc119 Is Expressed in Eosinophils and Mononuclear Cells**—Unc119/HRG4 was shown previously (25) to be predominantly expressed in the retina (Fig. 1C). However, its expression in hematopoietic cells was not studied. Because we cloned it from fetal liver, an early hematopoietic organ, we critically examined its expression in myeloid cells. We demonstrated the presence of its message in eosinophils by RT-PCR followed by Southern hybridization (Fig. 1A). We subcloned the RT-PCR-amplified Unc119 cDNA and confirmed its identity by sequencing (data not shown). We raised a rabbit polyclonal antibody and used it to detect the presence of Unc119 in eosinophils, mononuclear cells, and polymorphonuclear granulocytes by Western blotting. Immune serum but not the preimmune serum detected the Unc119 band, we depleted the anti-Unc119 antibody from the antibody-depleted serum (a-Unc119 depleted) (n = 3). C, comparison of Unc119 expression in various mouse organs. Tissue lysates (60 μg of protein) from retina, heart, liver, lung, and spleen were directly Western-blotted. The last lane shows the position of rUnc119. D, Unc119 interacts with IL-5Ra in the yeast. pLexA-5Ra and pB42AD-Unc119 constructs were transfected into the yeast EGY48 alone or together and plated first on selection and then on induction plates and were checked for the activation of the reporter gene lacZ. 53-T (pLexA-53 + pb42AD-T') and Pos (pLexA-53 fused to AD-T) were positive and Lam (pLexA-53 + pB42AD-Lam) was a negative control for the experiment (n = 2). E, Unc119 is associated with IL-5Ra but not with common β chain in eosinophils. Lysates from non-stimulated or IL-5-stimulated eosinophils were immunoprecipitated (IP) with either anti-IL-5Ra or anti-βc antibodies and Western-blotted with anti-Unc119 antibody (above). In the 1st lane rUnc119 (50 ng) was loaded alone in order to identify the position. The same membrane was stripped and reprobed with either anti-IL-5Ra (lower right) or with anti-βc (lower left) antibodies (n = 3).

**Detection**

detectable amounts of Unc119 were also present in other organs.

**Unc119 Is Associated with IL-5Ra**—We reconfirmed the interaction of IL-5Ra and Unc119 in the yeast. Cotransfection of the bait construct pLexA-5Ra and pB42AD-Unc119 into the yeast activated the reporter lacZ gene, indicating the interaction of IL-5Ra and Unc119. Neither the bait nor the Unc119 alone activated the reporter gene (Fig. 1D). Next we assessed
Unc119 Associates with Lyn and Hck—In the next step we investigated whether Unc119 was associated with SH2 and SH3 domain-containing signaling molecules. For this purpose we examined the association of Unc119 with Lyn, Hck, Itk, and phosphatidylinositol 3-kinase (PI3K) p110ε in lysates from both IL-5-stimulated and non-stimulated eosinophils (Fig. 1E), which is anticipated from the result of the yeast two-hybrid experiment. Under the same conditions, Unc119 did not associate with the common βc chain. The anti-βc antibody is a rabbit antibody and served as a control for the anti-Unc119 antibody.

Unc119 Has SH3- and SH2-binding Motifs—SH3-binding motifs contain the canonical PXXP sequence (12, 13) that is frequently flanked by a conserved arginine (Table I). Unc119 has one complete and one incomplete SH3-binding motif at the N terminus. It also has phosphorylation sites for SrcTKs and one SH2-binding motif (26), which also conforms to the consensus tyrosine phosphorylation site for SrcTKs, is located at the C terminus.

Unc119 Associates with Lyn and Hck—In order to examine the physical association of the native proteins in the cell, we immunoprecipitated Lyn and Western-blotted with the anti-Unc119 antibody (Fig. 3A). As a control for rabbit immunoprecipitating anti-Lyn antibody, we used the anti-βc antibody. Unc119 coprecipitated with Lyn but not with the control antibody (see also Fig. 1B). The bottom panel shows the amount of Unc119 that did not bind to Lyn and was detectable in the supernatant. The densitometric concentrations of rUnc119 (50 ng), Lyn-bound Unc119, and non-bound Unc119 are 1.4, 0.8, and 1.8 units, respectively. After correcting for the dilution factor of the supernatant (one-fifth of the whole cell lysate (WCL)), the membranes that were Western-blotted with anti-Hck and anti-PI3K antibodies were stripped and reprobed with anti-GST antibodies to confirm the presence and assess the quantity of GST or GST-Unc119 in the binding reactions (n = 4).

**TABLE I**

| SH2 motif | SH3 motif | Tyrosine phosphorylation |
|-----------|-----------|--------------------------|
| RXFXPXXP (class I) | (DE)X2Y-(DE)(FWP) | 26APIQPQP29; 26RQKPGP32 |
| (DE)X2Y-(DE)(FWP) | (R/K)X2Y-(DE)X2Y | 165EHEDFFP170; 165RMKIEHR170 |
| (R/K)X2Y-(DE)X2Y | 165KNTCEHIY179 |

α From Ref. 12.  
β Motif search at www.motif.genome.ad.jp. X indicates any amino acid; parentheses indicate acceptable residues; * indicates phosphorylation site.

In a next set of experiments, we examined whether the SH3 or the SH2 motif peptides derived from Unc119 bind to Lyn kinase. To this goal we synthesized a peptide encompassing the conserved RXFPPXP (residues 55–64) of the SH3 motif of Unc119 (P<sub>SH3</sub>) and a second peptide that had the conserved arginine and proline residues substituted with alanine (proline to alanine mutant, PP<sub>→A</sub>). Similarly we synthesized a third peptide encompassing the SH2 motif of Unc119 (residues 189–200) (P<sub>SH2</sub>) and a fourth peptide that had the tyrosine residue phosphorylated (P<sub>S</sub><sup>p</sup>). The association between the SH2 domains and their cognate SH2 peptide is typically mediated through phosphorylation of the tyrosine residue within the motif peptide. However, several groups (27–29) have reported that this association also occurs independent of phosphorylation. For example, the binding of the SH2 domain of SLAM-associated protein with non-phosphorylated signaling lymphocyte activation molecule (SLAM) peptides has been examined by crystallography, and the nature of the binding interaction has been fully delineated (29). The SH3 motif peptide (P<sub>SH3</sub>)}
Unc119, a Novel Activator of Src-type Kinases

Unc119 interacts with Lyn through SH2 and SH3 domains. A, Unc119 coprecipitates with Lyn. An aliquot of IL-5-stimulated eosinophil lysate was incubated with rabbit polyclonal anti-Lyn (α-Lyn) or with rabbit polyclonal anti-βc (α-βc) antibodies and immunoprecipitated (IP). Fifty nanograms of recombinant Unc119 (rUnc), the immunoprecipitated pellets (Pellet, upper panel), or one-fifth of each supernatant (Sup, lower panel) was separated with SDS-PAGE and Western-blotted with anti-Unc119 antibodies (n = 3). B, Unc119 associates with the SH2 and SH3 domains of Lyn. rUnc119 (2 μM) was incubated with 2 μM GST-fused recombinant domains (U, unique N terminus; SH2 and SH3, SH2 and SH3 domains) of Lyn, Grb2, SF, or Fyn in the lysis buffer, pulled down with glutathione-agarose, and Western-blotted with anti-Unc119 antibody (upper panel). One-fifth of each supernatant was separated on another gel and processed as above (lower panel) (n = 3). C, SH2 and SH3 motif peptides of Unc119 bind Lyn. An aliquot of leukocyte lysate was incubated with no peptide (–) or various concentrations of biotinylated SH3 (PSh3), mutated SH3 in which conserved arginine and proline residues were substituted with alanine (PSh3), (left panel), non-phosphorylated SH2 (PSh2) or phosphorylated SH2 (PPsh2) (right panel) motif peptides, precipitated with streptavidin-agarose, and Western-blotted with the anti-Lyn antibody (Pellet). One-fifth of the supernatant from each sample was also separated and processed as above to confirm the presence of Lyn in the lysates (lower panels, Sup) (n = 3).

Unc119 activates Lyn and Hck. The mechanism of activation of SrcTks following receptor stimulation is unclear. Because of its interaction with SH3 and SH2 domains, we studied the effect of Unc119 on Lyn and Hck activation. Initially, we examined the effect of Unc119 on Lyn autophosphorylation in an in vitro kinase assay. Unc119 stimulated autophosphorylation of Lyn in a dose-dependent manner with the optimum autophosphorylation occurring at a concentration between 7 and 70 nM Unc119 (Fig. 4A). To assess whether the increase in autophosphorylation was associated with enhanced catalytic activity, enolase was used as a Lyn substrate in a similar kinase assay. Unc119 induced enolase phosphorylation by Lyn in a dose-dependent manner (Fig. 4B). In order to confirm the kinase activity with another substrate, we used Sam68, a known substrate for Src (30). Unc119 was also able to induce the phosphorylation of recombinant Sam68 by Lyn (Fig. 4B).

Next, we investigated the effect of Unc119-derived motif peptides (PSh3, P–A, PSh2, and PPsh2) on Lyn activation. The PSh3 peptide induced autophosphorylation of Lyn (Fig. 4C, left panel) and kinase activity for enolase (Fig. 4C, right panel) at low concentrations (0.02 to 0.2 μM). In contrast, the P–A peptide induced negligible autophosphorylation and no substrate phosphorylation by Lyn. Previously, a SH3 motif peptide derived from CD2 was shown to induce Lyn activation at relatively high concentrations (1 mM) (31). We tested this peptide for Lyn activation under our assay conditions. This peptide induced auto phosphorylation of Lyn (Fig. 4D, upper panel) and kinase activity for enolase (Fig. 4D, lower panel) at ≥20 μM concentration. The Unc119-derived SH3 peptide compares favorably with the CD2 derived SH3 peptide in regard to Lyn activation.

Like the PSh3 peptide, the PSh2 and PPsh2 peptides induced Lyn autophosphorylation at relatively low concentrations (Fig. 4E, left panel). Both SH2 peptides were also able to induce substrate enolase phosphorylation by Lyn, although the PSh2 peptide seems to be more effective than the PPsh2 peptide (Fig. 4E, right panel). An irrelevant peptide (Pc) derived from Unc119 did not induce enolase phosphorylation by Lyn (Fig. 4F).

To assess whether Unc119 is a specific activator of SrcTks, we examined the effect of Unc119 and its motif peptides on the kinase activity of Hck, Itk, and p38 mitogen-activated protein kinase (Fig. 5). We chose Itk as a non-Src tyrosine kinase and p38 as a serine/threonine kinase. Unc119, the PSh2 and PPsh2 but not the P–A or PPSH2 peptide increased Hck activation (Fig. 5A). In contrast, neither Unc119 nor the peptides modified the catalytic activity of Itk or p38 (Fig. 5, B and C). Enolase and transcription factor ATF-2 were used as the substrates for Itk and p38, respectively. Itk from pervanadate-treated mononuclear cells was active and phosphorylated enolase (Fig. 5B). Similarly, p38 from granulocyte-macrophage colony-stimulat-
Unc119, a Novel Activator of Src-type Kinases

Unc119, also known as HRG4 for human retinal gene protein 4, was originally cloned as a retina-specific gene (25). It had been localized in ribbon synapses of retina (35). It has 57% homology to the unc-119 gene from Caenorhabditis elegans, which is expressed in the neural tissue and is involved in feeding, locomotion, and chemosensation of the nematode (36). Recently, a single patient with cone-rod dystrophy who expressed a truncated form of HRG4 has been identified (37). This patient is heterozygous, and it is not clear from the report whether this patient displays any other abnormalities. Rhodopsin promoter-driven overexpression of this truncated HRG4 in the retina causes its degeneration in mice (37). The foregoing observation is exciting and clearly indicates an important role for HRG4/Unc119 in retina. However, the exact function of HRG4/Unc119 in retina and other organs remains unknown.

Crystallographic studies predict that the binding of the SH3 domain to an external SH3 ligand would release the linker region leading to a conformational change in the kinase domain and its activation. Indeed, studies using human immunodeficiency virus Nef, an exogenous SH3 ligand, have demonstrated robust activation of SrcTKs in vitro (14). Nef is not the only SH3 ligand that activates SrcTKs. Two cell surface receptors, CD28 and CD2, have proline-rich sequences that conform to the SH3-binding motif. In support of our findings both CD28 and CD2 activate Lck and Fyn, respectively, in an SH3 motif-dependent manner (31). HeterotrimERIC guanine nucleotide-binding regulatory protein (G protein) can directly activate Src and Hck (38). Unc119 is not the first molecule that binds an Src-type kinase at two sites and regulates its function. It has been shown that p130Cas (Csk-associated substrate) binds Src kinase through its SH2 and SH3 domains (39). The binding through the SH3 domain activates Src. A mutation in the SH3-binding motif of p130Cas causes significant reduction in Src kinase activity (39). We have seen activation of Lyn by both SH2 and SH3 peptides. However, higher concentrations of

**DISCUSSION**

Lyn, a member of SrcTKs, plays an important role in many hematopoietic cells, especially in B cells (33), mast cells (34), and eosinophils (17). The activation of eosinophils through the IL-5 receptor is critically dependent upon Lyn kinase (19–21). The mechanism of activation of Lyn following receptor stimulation is unknown. We have cloned Unc119 using the IL-5Rα subunit as the bait in the yeast two-hybrid screening. Unc119 has SH2- and SH3-binding motifs. It associates with IL-5Rα and Lyn kinase in eosinophils. More importantly, it activates Lyn kinase in an SH2- and SH3-dependent manner. Unc119 also binds to Hck but not to Itk or P38Kp110y. Furthermore, it does not interact with the SH2 or SH3 domains of Grb2 and Shc, respectively. The SH3 motif peptide of Unc119 activates both Lyn and Hck suggesting that a common principle is applicable to Unc119 interaction with SrcTKs. Interestingly Itk, which has both SH2 and SH3 domains, does not bind to nor is activated by Unc119. The results suggest the existence of microspecificity of SH3 interaction with their ligands.

Immunoprecipitation experiments suggest that only a small fraction of Unc119 is associated with Lyn in vivo (Fig. 3A). The result may suggest that this association is physiologically not important. However, because the binding of Unc119 leads to the activation of Lyn, it is likely that Unc119 associates with Lyn only transiently. Indeed, the association of Unc119 with Lyn increases after IL-5 stimulation (Fig. 5A). The Unc119-associated Lyn shows increased kinase activity. More importantly, the entire activable and detergent-soluble Lyn fraction is associated with Unc119 following IL-5 stimulation.

Empowerment of Unc119 signaling is critical to maintain eosinophil survival (21). In order to assess the biological relevance of Unc119—IL-5 Stimulates Unc119 Association with Lyn and Its Kinase Activity—In order to assess the biological relevance of Unc119 in IL-5 signaling, we stimulated eosinophils with IL-5 and examined the association of Unc119 with Lyn and Unc119-bound Lyn activity. IL-5 enhanced the association of Unc119 with Lyn and its catalytic activity (Fig. 6A). The amount of kinase activity that coprecipitated with Unc119 was similar to that associated with the anti-Lyn immunoprecipitate.

Transduced Unc119 Activates Lyn and Promotes Cell Survival in the Absence of Growth Factors—Next, we examined whether Unc119 modulated SrcTK-dependent cellular functions. IL-5 regulates eosinophil survival by activating SrcTKs (19–21) among others. The eosinophil is a terminally differentiated non-proliferating cell and cannot be easily transfected with expression vectors or propagated in selection medium. To overcome this problem we used a novel protein transduction reagent called Chariot. This reagent combines the nuclear localization sequences with the retroviral gp41 fusion domain (32). In order to test its capacity to transduce protein into cells, we incubated cells with FITC-labeled ovalbumin in the presence or absence of Chariot. A significant quantity of FITC-labeled ovalbumin was internalized in the presence of Chariot (Fig. 6B). Next, we examined the effect of Unc119 transduction on Lyn kinase activation in eosinophils. Unc119-transduced cells show increased Lyn activation (Fig. 6C), which was comparable with or better than that stimulated by IL-5 (10–12 μM). The likely explanation for this strong activation is that we have used a relatively high concentration of Unc119 (5 μg/106 cells) in order to maximize the outcome and to mimic protein overexpression that is typically achieved with expression plasmids. By using this experimental model we studied the effect of Unc119 on eosinophil survival. Unc119 significantly prevented eosinophil apoptosis (Fig. 6D, annexin V- and annexin V+ propidium-positive cells) and increased survival (annexin V and propidium negative cells). In the absence of growth factors 9–16% eosinophils were alive on day 3. The number of live eosinophils was dramatically improved to 70% in Unc119-transduced cells. The effect of Unc119 (5 μg/106 cells) was comparable with that seen with IL-5 (10–12 μM). Based upon the results of the kinase experiment (Fig. 6C), we speculate that the transduced Unc119 promoted eosinophil survival by activating Lyn kinase. However, we understand that the effect of interaction of Unc119 with other unidentified signaling molecule(s) in promoting eosinophil survival cannot be ruled out.

Next we studied the effect of the anti-Unc119 antibody on IL-5-stimulated eosinophil survival. Eosinophils were transduced with the affinity-purified anti-Unc119 antibody in the presence of Chariot and then examined for IL-5-stimulated eosinophil survival. The anti-Unc119 antibody (39 versus 84% with IL-5) but not a control IgG (73 versus 84% with IL-5) partially inhibited IL-5-induced eosinophil survival suggesting that Unc119 is physiologically relevant to IL-5 signaling (Fig. 7).

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the SH2 peptide are needed to activate Lyn. We speculate that the SH3-binding motif may be physiologically more relevant than the SH2-binding motif for Lyn activation.

Unc119 and Unc119-derived motif peptides induce autophosphorylation of Lyn as well as kinase activity for the substrate. However, we have observed that autophosphorylation occurs at

Fig. 6. A, IL-5 stimulates Unc119-associated Lyn kinase activity. Eosinophils were incubated with buffer (−) or IL-5 (10^{-10} M) (+) for 5 min, immunoprecipitated (IP) with the preimmune, anti-Unc119, or anti-Lyn antibodies followed by kinase assay using enolase (Enol) as a substrate. *, Lyn immunoprecipitate was loaded alone in the absence of enolase in the 1st lane in order to identify the position of Lyn kinase. One of two separate experiments is shown. B, protein transduction by Chariot. Cells were incubated with OVA-FITC ± chariot and then examined by flow cytometry. C, activation of Lyn by Chariot-transduced Unc119. Eosinophils were incubated with Chariot and Unc119 (5 μg/10^6 cells) ± IL-5 (10^{-10} M) and then lysed, immunoprecipitated with anti-Lyn antibody, followed by kinase assay using enolase as the substrate. One of two separate experiments is shown. D, effect of Unc119 on eosinophil apoptosis. Eosinophils were incubated with chariot ± Unc119 or human serum albumin (HSA) (both at 5 μg/10^6 cells) and then cultured ± IL-5 (10^{-10} M). Eosinophil apoptosis was assessed by flow cytometry on day 3 following staining for annexin V and propidium iodide. Results of one of two separate experiments are shown.
a concentration of Unc119 that is frequently lower than required for the induction of kinase activity. The discrepancy between autophosphorylation and substrate phosphorylation has been noted previously by other investigators (40). The interplay between autophosphorylation and kinase activity of SrcTKs is very complex and is dependent upon multiple factors including divalent cation and ATP concentrations and affinity for substrates and activators. This complex process was recently investigated by Sun et al. (41), who demonstrated that autophosphorylation of Src is inhibited during the peak of substrate phosphorylation.

One of the important functions of IL-5 and other growth factors is to delay eosinophil apoptosis and prolong eosinophil survival. Lyn kinase has been shown previously (19, 20) to play a non-redundant role in this process. Inhibition of Lyn blocks eosinophil survival. When transduced into eosinophils Unc119 activated Lyn kinase and inhibited eosinophil apoptosis in the absence of growth factors. The anti-Unc119 antibody blocks eosinophil survival suggesting that Unc119 is important for Lyn activation in vivo. In other work\(^2\) we have demonstrated that overexpression of Unc119 induces activation of Lck and Fyn in T cells. Unc119-deficient cells are unable to activate Lck and Fyn following T cell stimulation. As a consequence these cells are unable to produce IL-2 and proliferate poorly. Thus, Unc119 appears to be a receptor-associated activator of SrcTKs. We believe that in eosinophils Unc119 and Lyn form an inactive complex with IL-5R\(\alpha\) under basal conditions, and Unc119 is inaccessible to the Lyn SH3 domain. Receptor activation changes the conformation allowing the association of Unc119 with the Lyn SH3 domain and leading to kinase activation. The association through the SH2 domain may have an additive effect. By activating SrcTKs Unc119 may provide a novel signal-generating mechanism for receptors and, therefore, may have broad biological relevance.

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Identification of UNC119 as a Novel Activator of SRC-type Tyrosine Kinases
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