Expression of Saxiphilin in Insect Cells and Localization of the Saxitoxin-Binding Site to the C-Terminal Domain Homologous to the C-Lobe of Transferrins†

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ABSTRACT: Saxiphilin is a plasma protein from the bullfrog (*Rana catesbeiana*) that is homologous to transferrin. Most known transferrins contain two binding sites for Fe^{3+}/HCO_3^-, one in each of two homologous domains called the N-lobe and C-lobe. However, native saxiphilin does not bind Fe^{3+} but stoichiometrically binds one molecule of the neurotoxin saxitoxin (STX) with a dissociation constant (K_D) of ~0.2 nM. To pursue structural analysis of the STX binding site, cDNA encoding saxiphilin was used to construct a baculovirus expression vector that directs synthesis and secretion of a ~92-kDa recombinant saxiphilin protein (R-sax) in cultured insect cells. Culture medium harvested from infected cells contained 25−67 pmol of [^{3}H]STX binding sites/mL with a K_D of 0.22 nM. The kinetics and pH dependence ($pK_{0.5}$ = 5.4) of [^{3}H]STX binding to R-sax are similar to native saxiphilin, implying proper folding and functional activity. Another baculovirus expression vector was constructed to encode a deletion mutant of saxiphilin consisting of the first 20 N-terminal residues containing the secretory signal sequence spliced to the C-terminal, 361-residue fragment homologous to the C-lobe domain of transferrins. This vector directed the secretion of a ~38-kDa derivative of saxiphilin (C-sax) that was recognized by anti-saxiphilin antibody. C-sax also exhibited [^{3}H]STX binding activity with a lower affinity K_D of ~0.9 nM, a 4-fold faster dissociation rate for [^{3}H]STX than native saxiphilin, and a pH dependence ($pK_{0.5}$ = 5.7) similar to R-sax ($pK_{0.5}$ = 5.4). These results establish that the binding site for STX and residues that determine the pH dependence of toxin binding are located within the C-lobe domain of saxiphilin.

Saxiphilin is a soluble protein found in plasma and tissues of various animals that is characterized by high affinity for saxitoxin (STX),[1] a tricyclic neurotoxin that blocks voltage-sensitive sodium channels (Doyle et al., 1982; Mahar et al., 1991). Purification and partial sequencing of the native protein (Li & Moczydlowski, 1991) led to the isolation of cDNA encoding saxiphilin from the bullfrog (*Rana catesbeiana*) (Morabito & Moczydlowski, 1994, 1995). Surprisingly, the primary sequence of saxiphilin was found to be homologous to transferrins, a family of high-affinity (K_D ~ 10^{-20} M) Fe^{3+}-binding proteins [reviewed in Crichton (1991) and Welch (1992)]. For example, the amino acid sequence of saxiphilin is 51% identical to serum transferrin of *Xenopus laevis* (African clawed frog) and 42% identical to human lactoferrin. However, saxiphilin differs from the transferrin family by substitutions in 9 of 10 highly conserved residues directly involved in binding of Fe^{3+} and an anion cofactor (HCO_3^-) in the two metal-binding sites of transferrins (Anderson et al., 1989; Bailey et al., 1988). These latter differences account for the inability of saxiphilin to bind Fe^{3+} (Li et al., 1993). Bullfrog saxiphilin is also distinguished by a unique insertion of 143 residues composed of a tandem duplication which contains two copies of a recognized protein module known as a type 1 thyroglobulin domain (Malthiery & Lissitzky, 1987; Morabito & Moczydlowski, 1994, 1995).

In humans, Fe^{3+}-binding members of the transferrin family include serum transferrin, melanotransferrin, and lactoferrin. Serum transferrin is the major iron carrier and transport protein in vertebrates and is an important determinant of cell growth. It is internalized by binding to a cell surface receptor followed by endocytosis, acidification of the endosome compartment, release of Fe^{3+}, and recycling of apotransferrin to the cell surface (Dautry-Varsat, 1986; Thorstensen & Romslo, 1990). Melanotransferrin is abundantly expressed on the surface of melanoma cells (Rose et al., 1986) and contains only one functional Fe^{3+}-binding site in its N-terminal domain (Baker et al., 1992). Melanotransferrin does not appear to mediate uptake of Fe^{3+} (Richardson & Baker, 1992). Its exact function is unknown. Lactoferrin is present in milk and other secretions and is also found within secondary granules of neutrophils (Bullen, 1987). Along with transferrin, lactoferrin inhibits microbial infections by limiting the availability of free iron (Griffiths & Bullen, 1987). These two proteins are also considered to provide an important protection against the potential toxicity of free Fe^{3+}/Fe^{2+} ions, which mediate the production of hydroxyl free radical via the Fenton reaction (Crichton, 1991). Lactoferrin appears to possess a variety of other regulatory

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[1] Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; PCR, polymerase chain reaction; pfu, plaque-forming units; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; STX, saxitoxin; Tris, tris(hydroxymethyl)aminomethane.

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activities that include modulation of the immune system. Recently, lactoferrin has been documented to bind to specific DNA sequences and activate the transcription of a reporter gene (He & Furmanski, 1995).

The structural homology between saxiphilin and transferrins leads to the question of whether any of the known or proposed paradigms of transferrin function apply to saxiphilin. For example, does saxiphilin function as a toxin defense mechanism, growth factor, intracellular ligand-delivery vehicle, transcription factor, etc.? To address these questions, it is necessary to establish methods for production of the recombinant protein. This would provide a readily available source of saxiphilin for structural analysis, investigation of its cellular physiology, and possible application in detection assays for STX. For this purpose, we have constructed a baculovirus expression vector that can be used for the production of recombinant saxiphilin by cultured insect cells. In this paper, we describe the [^{3}H]STX binding activity of the recombinant protein. In order to define the location of the STX binding site, this system was used to express a deletion fragment of saxiphilin consisting of only the C-terminal domain homologous to the C-lobe of transferrins. This truncated protein binds [^{3}H]STX in a manner similar to that of the whole protein, demonstrating that the C-terminal half of the molecule contains the toxin binding site. A preliminary report of this work has been published in abstract form (Morabito et al., 1995).

MATERIALS AND METHODS

Materials. High Five insect cells, Sf9 insect cells, the pBlueBac III baculovirus transfer vector, and the pCR II vector were purchased from Invitrogen (San Diego, CA). Grace's insect cell medium, Sf-900 serum-free insect cell medium, heat-inactivated fetal bovine serum, and 4% agarose gel with Bluo Gal were from GibcoBRL (Grand Island, NY). The BaculoGold insect cell transfection kit was obtained from Pharmingen (San Diego, CA). [^{3}H]STX and the ECL Western blot detection kit were purchased from Amersham (Arlington Heights, IL). *Taq* polymerase (Gene Amp) was from Cetus (Norwalk, CT). Plasmid constructs were propagated in *Escherichia coli* strains DH5α (GibcoBRL) or SURE (Stratagene, La Jolla, CA). Oligonucleotides were synthesized by the Yale Protein and Nucleic Acid Chemistry Facility.

Sequence Information. In the course of this study, a few sequencing errors were discovered in the originally reported cDNA sequence of saxiphilin (Morabito & Moczydlowski, 1994). In the revised translation, the secreted form of saxiphilin is 825 residues in length instead of 826 residues as reported previously. Residue numbers of saxiphilin given here refer to the corrected sequence summarized in Morabito and Moczydlowski (1995) and updated in the Genbank data base (Accession Number U05246).

Baculovirus Expression Vector for Recombinant Saxiphilin. Excision of a pBluescript SK− plasmid containing saxiphilin cDNA was accomplished by coinfection of *E. coli* with Lambda ZAP II phage containing the previously isolated clone (Morabito & Moczydlowski, 1994) and helper phage according to the Strategene protocol. The pBluescript SK−/saxiphilin plasmid was digested with *Pvu*I, which does not cut the saxiphilin insert. This linearized DNA was used as a template to amplify saxiphilin cDNA by PCR using a T3 sense primer (5′-ATTAACCCTCACTAAAG-3′) and an antisense primer, anti-XP (5′-CTGCAGTCTAGAGAAGATCAACGTGCCA-3′), that was designed to match a 16-nucleotide sequence in the 3′ untranslated region of saxiphilin cDNA linked to restriction sites for *Xba*I and *Pst*I. The PCR contained ~0.1 μg of template DNA, 1 μM 5′ and 3′ primers and was carried out for 30 cycles (1 min at 94 °C, 2 min at 50 °C, and 2.5 min at 72 °C). The ~2.7-kb PCR product was digested with *Pst*I and subcloned into the *Pst*I-cut pBlueBac III transfer vector and also back into *Pst*I-cut pBluescript SK−. The latter saxiphilin cDNA in pBluescript SK− was completely sequenced by the method of Sanger et al. (1977) to determine whether any errors were introduced by PCR. Insect Sf9 cells were coinfected with the pBlueBac III/saxiphilin vector and modified linear baculovirus DNA according to directions of the Pharmingen BaculoGold transfection kit to generate infective recombinant baculovirus containing saxiphilin cDNA inserted downstream of the polyhedrin promoter. A single virus plaque was isolated and propagated by conventional methods (Summers & Smith, 1987; O'Reilly et al., 1994).

Baculovirus Expression Vector for the C-Lobe of Saxiphilin. The *Pvu*I-cut pBluescript SK−/saxiphilin plasmid was first used as a template to amplify saxiphilin cDNA coding for residues 466−825 by PCR. This reaction used a sense primer (5′-CATCTTCCATCCAAAAATAAAGTGCGG-3′) corresponding to the cDNA sequence of saxiphilin residues 466−474 and a T7 antisense primer (5′-AATACGACTCACTATAG-3′). Using the product of this reaction as a template, two consecutive rounds of PCR (1 min at 94 °C, 2 min at 42 °C, and 2 min at 72 °C, 30 cycles) were then performed to add the N-terminal secretory signal sequence of native saxiphilin. The first round of PCR used a sense primer, 5′-CTTGAGCTTTGCGGCAGCACATCTTCCATCC-3′, and the anti-XP antisense primer described above. The second round of PCR used the sense primer 5′-CGATCTGCAGATGGCTCCGACTTTCCAAACAGCTCTGTTTTTCACCATCATTAGCTTGAGCTTTGCGGC-3′ and the anti-XP antisense primer. The resulting product consisted of cDNA coding for amino acid residues −19 to 1 linked to 465−825 of native saxiphilin that is flanked by restriction sites for *Pst*I. The final PCR product was subcloned into the TA cloning site of pCR II and completely sequenced. This confirmed an open reading frame with one nonsilent, PCR-generated mutation (corresponding to mutation of Ser616 in native saxiphilin to Pro). The final cDNA coding for saxiphilic C-lobe was excised from pCR II with *Pst*I and inserted into the *Pst*I cloning site of the pBlueBac III vector. This construct was used to generate an infectious recombinant baculovirus as described above for recombinant saxiphilin.

Insect Cell Culture and Production of Recombinant Saxiphilin. For routine production of baculovirus, insect Sf9 cells (derived from *Spodoptera frugiperda* ovarian cells) were grown as adherent cells in Grace's medium supplemented with 10% fetal bovine serum. To isolate recombinant virus, agarose plugs from single plaques were resuspended in 1 mL of FBS-supplemented Grace's medium and used to infect ~2 × 10^6 Sf9 cells. Virus-containing medium was titered by plaque assay (Summers & Smith, 1987) using 4% agarose gel with Bluo Gal to reveal blue-colored plaques of recombinant virus. Recombinant virus stock containing ~5 × 10^7 to 5 × 10^8 pfu/mL was produced by amplification in

adherent cultures of Sf9 cells. For small-scale production and characterization of secreted recombinant saxiphilin, adherent cultures of ~5 × 10^6 insect High Five cells (derived from *Trichoplusia ni* ovarian cells) were grown in Sf-900 serum-free medium and infected with virus stock at a multiplicity of ~5 pfu/cell. The cell culture medium was collected 3 days after infection, supplemented with protease inhibitors (1 μM leupeptin, 1 μM pepstatin, and 5 mM EDTA), and clarified of cell debris and virus by microcentrifugation (5 min at 16000*g*). The resulting supernatant was directly analyzed by SDS−PAGE, immunoblot assay, and [^{3}H]STX binding assays. Large-scale production of recombinant saxiphilin was performed by the National Cell Culture Center (Minneapolis, MN). One-liter suspension cultures of High Five cells were grown to a density of ~1 × 10^6 cells/mL in serum-free HyQ CCM3 medium (Hyclone) and infected with recombinant baculovirus at a multiplicity of 2−5 pfu/cell. Three days after infection, the cell culture medium was harvested, centrifuged, supplemented with inhibitors (0.01% phenylmethanesulfonyl fluoride, 3 mM NaN_3, and 5 mM EDTA), and shipped on ice to our laboratory.

SDS−PAGE and Immunoblot Analysis. Samples (15 μL) of serum-free cell culture medium were subjected to SDS−PAGE (Laemmli, 1970) using a 10% polyacrylamide gel. The slab gel was electroblotted onto a nitrocellulose membrane (0.2 μm) using a Sartoblot IIS semidry electroblotting apparatus (Sartorius). The membrane was preincubated for 10 min with Blotto (5% nonfat dry milk, 20 mM Tris-HCl, pH 7.3, 150 mM NaCl, and 0.1% Tween 20) and then incubated for 1 h at 22 °C with 1:100 dilution (in Blotto) of affinity-purified rabbit antibody against native saxiphilin (Li & Moczydlowski, 1993). Following three consecutive 5-min washes in Blotto, the membrane was incubated with 1:1000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG antibody for 1 h at 22 °C. After two consecutive 10-min washes in Blotto, followed by two consecutive washes in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$, immunoreactive protein complexes were visualized by chemiluminescence detection according to instructions of the ECL Western blotting kit (Amersham).

[^{3}H]STX Binding Assays. Measurements of equilibrium [^{3}H]STX binding, Scatchard analysis, association and dissociation kinetics, and assay of the pH dependence of [^{3}H]-STX binding to recombinant saxiphilin were performed essentially as previously described for native saxiphilin (Llewellyn & Maczydlowski, 1994). Bound [^{3}H]STX was separated from free toxin by rapid passage of 100-μL aliquots over minicolumns of AG50W-X2 resin (100−200 mesh, Tris+ form) that were preequilibrated with 100 mM Tris-HCl, pH 7.4, and 10 mg/mL bovine serum albumin. The standard incubation buffer for the binding assay was 20 mM Mops-NaOH, pH 7.4, and 200 mM NaCl. In the binding experiments of Figures 2, 3, 5, and 6, the assay mixture contained either 5 μL (R-sax) or 8 μL (C-sax) of undiluted cell culture supernatant containing the respective forms of recombinant saxiphilin in 250 μL of assay volume. The pH titration of Figure 6 was performed using a buffer containing 200 mM NaCl and 20 mM Tris/10 mM Mes/10 mM acetic acid adjusted with tetramethylammonium hydroxide or HCl as designed to maintain a relatively constant ionic strength in the range of pH 4−9 (Ellis & Morrison, 1982). Data were corrected for nonspecific [^{3}H]STX binding by subtraction of blank assays carried out in the presence of 10 μM nonradioactive STX. All [^{3}H]STX binding assays were performed at 0 °C by sample incubation on ice. The working specific activity of [^{3}H]STX used in these experiments was 37 700 cpm/pmol.

RESULTS

Predicted Domain Structure of Saxiphilin. The primary sequence of saxiphilin was aligned with that of human lactoferrin using the GAP program (gap weight 3.0, length weight 0.1) of the GCG Wisconsin sequence analysis package (version 8). As described previously (Morabito & Moczydlowski, 1994), this alignment is characterized by homology throughout the two sequences (42% identity, 63% similarity) except for an insertion of 143 residues in saxiphilin. The alignment was used to predict structural domains of saxiphilin (Figure 1) corresponding to those of lactoferrin previously identified in an X-ray crystal structure (Anderson et al., 1989). The residue numbering in Figure 1 reflects recent corrections to the saxiphilin sequence (Morabito & Moczydlowski, 1995). Saxiphilin is composed of an N-terminal secretory signal peptide (residues −19 to −1) followed by 825 residues of the mature protein. Saxiphilin can be subdivided into an N-lobe domain (residues 1−461) and a C-lobe domain (residues 473−825), which coincides with an internal duplication (~39% identity) that is found in all members of the transferrin family. These two lobes correspond to distinct globular domains with a similar fold in the lactoferrin crystal structure (Anderson et al., 1989). The presumed N- and C-lobe domains are linked by a short connecting peptide (residues 462−472 in saxiphilin). In lactoferrin, this connecting peptide is an α-helical segment that connects the N- and C-lobes. From the known pattern of disulfide bonding in transferrins, saxiphilin is expected to contain at least six conserved disulfide linkages in the N-lobe and eight in the C-lobe that are numbered a−f and a′−h′, respectively, in Figure 1 at the position of corresponding cysteine residues. Structural analysis of lactoferrin has also identified two globular subdomains in each lobe, known as N1/N2 and C1/C2, that appose to form a cleft where each of the two Fe^{3+}/HCO_3^- binding sites is located. The presumed linear sequence map of these subdomains in saxiphilin is also identified in Figure 1 on the basis of their homologous location in lactoferrin. In addition, saxiphilin contains a unique 143-residue insertion that occurs at a position between subdomains N1 and N2, which forms a hinge region in lactoferrin. This saxiphilin insertion is itself an internal duplication, containing two homologous modules (labeled Thyr-1A and Thyr-1B in Figure 1) that each contain a type 1 repetitive element of thyroglobulin (Mathiery & Lissitzky, 1987).

Previous work showed that equilibrium binding of [^{3}H]-STX to saxiphilin exhibits a pH dependence similar to that of Fe^{3+} binding to transferrins (Llewellyn & Moczydlowski, 1994). This apparently conserved aspect of the ligand-binding mechanism suggested that STX may bind in a cleft between subdomains N1/N2 or C1/C2 in an aqueous cavity analogous to the location of one of the two Fe^{3+}/HCO_3^- sites in transferrins. Since the opening and closing of this cleft in lactoferrin is mediated by the hinge region (Gerstein et al., 1993), the obstructing presence of the large 143-residue insertion at this location in the N-lobe of saxiphilin suggested that the C-lobe of saxiphilin is the most likely candidate for the STX binding site. Alternatively, it might be supposed

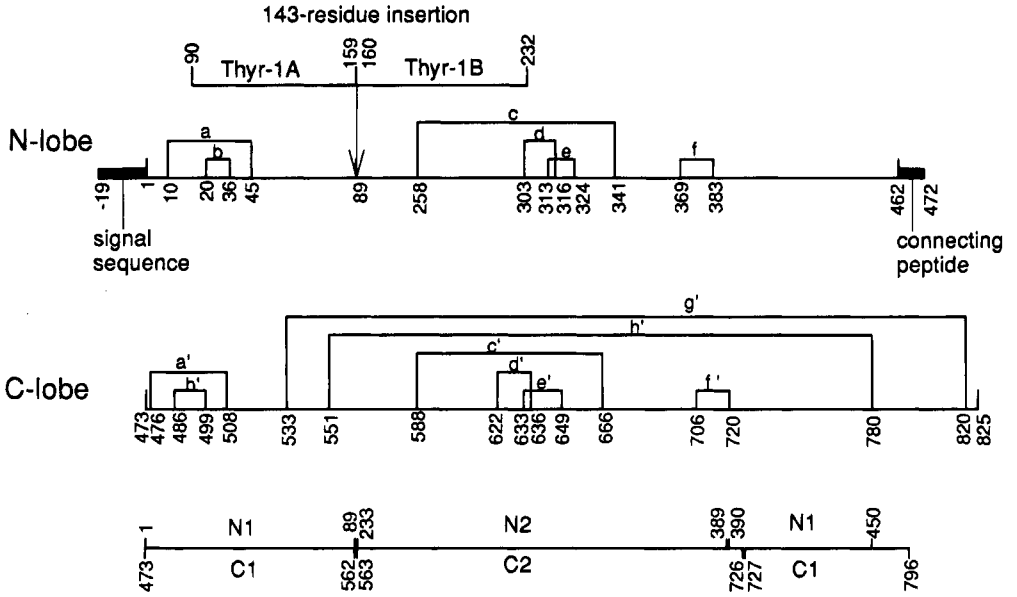

FIGURE 1: Diagram of the linear sequence of bullfrog saxiphilin showing the location of conserved disulfide bonds and N- and C-lobe structural domains predicted on the basis of sequence alignment with human lactoferrin. Numbers refer to the saxiphilin amino acid sequence (Morabito & Moczydlowski, 1994, 1995) and residues pairs a−f and a′−h′ indicate predicted disulfide bonds. Saxiphilin residues 90−232 containing two type 1 thyroglobulin domains (Thyr-1A and 1B) are shown as an insertion in the N-lobe. The bottom line shows the relative location of saxiphilin sequences corresponding to predicted N1/N2 and C1/C2 subdomains of the N-lobe and C-lobe, respectively.

that the 143-residue insertion in the N-lobe defines the STX binding site. To test these hypotheses, we engineered expression vectors for production of whole recombinant saxiphilin and a truncated form of saxiphilin corresponding to the presumed C-lobe domain in Figure 1.

Construction of Baculovirus Expression Vectors. To construct a baculovirus expression vector for recombinant saxiphilin (R-sax), the previously cloned, full-length saxiphilin cDNA sequence was amplified by PCR from the original clone in pBluescript SK− and inserted into the baculovirus transfer vector pBlueBacIII. Insect Sf9 cells were cotransfected with this transfer vector and modified linear baculovirus DNA. Clones of the recombinant, infectious virus were isolated and purified as described in Materials and Methods. Construction of a baculovirus expression vector for recombinant C-lobe of saxiphilin (C-sax) likewise involved PCR amplification of a DNA sequence composed of a synthetic oligonucleotide sequence encoding amino acid residues −19 to +1 of saxiphilin linked in frame to cDNA encoding saxiphilin residues 465−825. The amplified C-sax DNA sequence was inserted into pBlueBacIII, and recombinant virus was isolated by the same cotransfection procedure used for R-sax.

Sequencing of the PCR product showed that no errors had occurred in the coding sequence for R-sax; however, two base changes were observed for C-sax. One of these changes was a C → T substitution in the 3′ position of the TCC codon, corresponding to Ser-469 in native saxiphilin, which did not change the amino acid translation. The second change was a T → C substitution in the 5′ position of the TCC codon corresponding to Ser-616 in native saxiphilin, which resulted in a mutation of this residue to Pro. Since the baculovirus vectors for R-sax and C-sax were designed to include the native secretory signal sequence of the bullfrog protein (residues −19 to −1 of saxiphilin), we tested for expression by assaying the culture medium of Sf9 and High Five insect cells infected with both types of recombinant virus for [^{3}H]-STX binding activity.

Expression of Recombinant Saxiphilin and the C-Lobe of Saxiphilin. There was no detectable [^{3}H]STX binding activity in control medium taken from culture flasks of the uninfected insect cell lines, Sf9 and High Five, but such activity was observed within 1 day after infection with recombinant baculovirus coding for R-sax and C-sax. The [^{3}H]STX binding activity measured in the infected cell medium increased as a function of time and reached a maximal level at approximately 3 days after infection (not shown), when characteristic cell lysis occurs in the baculovirus infection cycle (Summers & Smith, 1987; O'Reilly et al., 1994). Figure 2A shows an example of a binding titration performed with a fixed amount of medium collected from Sf9 cells infected with R-sax baculovirus. In this experiment, increasing concentrations of [^{3}H]STX up to 10 nM were incubated with 5 μL of culture medium in a standard assay volume of 250 μL and bound [^{3}H]STX was separated from the free toxin on small cation-exchange columns. The data indicates the presence of a saturable component of [^{3}H]STX binding in the culture medium. In contrast, only a small, linear component corresponding to nonspecific background in the column assay was observed in the presence of excess unlabeled STX. Scatchard plots of specific [^{3}H]STX binding to culture medium from cells expressing R-sax and C-sax (Figure 2B) were consistent with a single class of sites with K_D values of 0.22 ± 0.01 nM and 0.93 ± 0.11 nM for R-sax and C-sax, respectively. Taking into account assay dilution factors, the maximal binding activity in the experiment of Figure 2B is equivalent to 25 pmol of [^{3}H]STX binding sites/mL of culture medium for R-sax and 21 pmol/mL for C-sax. For 13 different large-scale trials of R-sax expression in 1-L suspension cultures of High Five cells, the mean [^{3}H]STX binding activity of the harvested culture medium was 40 ± 13 pmol/mL (±SD). Using a value of 91 000 for the protein molecular weight of saxiphilin, the observed range of binding activity corresponds to a potential yield of 2.2−6.1 mg of saxiphilin/L.

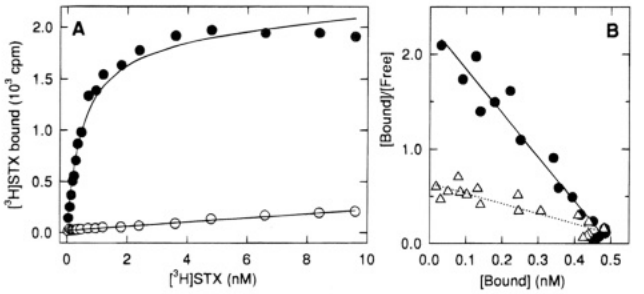

FIGURE 2: [^{3}H]STX binding titration of culture medium from baculovirus-infected insect cells. (A) Raw data for binding of [^{3}H]-STX to medium collected from High Five cells expressing R-sax assayed in the absence (●) and presence (○) of 10 μM unlabeled STX to assess nonspecific binding. Data points are the mean of duplicate samples. (B) Scatchard plots of [^{3}H]STX binding to medium from High Five cells expressing either R-sax (●, K_D = 0.22 nM) or C-sax (△, K_D = 0.93 nM).

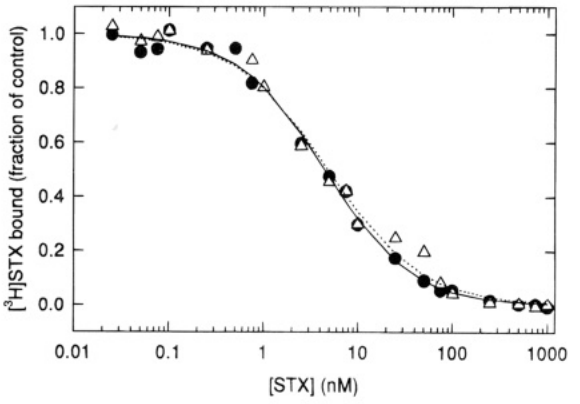

FIGURE 3: Competition of bound [^{3}H]STX with unlabeled STX. An aliquot of culture medium from High Five cells expressing R-sax (●) or C-sax (△) was assayed for specific binding in the presence of 5 nM [^{3}H]STX and various concentrations of STX. Specific binding is expressed as a fraction of the control value in the absence of unlabeled STX. Data points are the mean of duplicate samples. Data are fit to the equation $y = K_{0.5}^{n'}/(K_{0.5}^{n'} + [STX]^{n'})$ using the parameters, for R-sax (solid line), $K_{0.5}$ = 4.4 nM and n' = 0.94, and for C-sax (dotted line), $K_{0.5}$ = 4.8 nM and n' = 0.88.

To further characterize the homogeneity of the binding activity, a ligand competition assay was performed by titration of unlabeled STX in the presence of a fixed concentration of [^{3}H]STX. For medium taken from cells expressing C-sax and R-sax, the ligand competition assay was consistent with one class of STX binding sites as indicated by Hill coefficients close to 1.0 (Figure 3, legend). In such experiments, the K_D of the competitor ligand (STX) can be estimated from the concentration of STX at 50% displacement, $K_{0.5}$, according to the relationship $K_{0.5} = K_D(1 + [STX^*]/K_D^*)$, where [STX*] is the free concentration of [^{3}H]STX and K_D* is the equilibrium dissociation constant for [^{3}H]STX independently determined by the direct Scatchard analysis of Figure 2B. This use of this equation gives K_Ds of 0.18 nM and 0.75 nM for STX binding competition to R-sax and C-sax, respectively. Aside from demonstrating a single class of binding sites, the close agreement between the calculated K_D values for unlabeled STX and those for [^{3}H]STX measured by Scatchard analysis confirms that the concentration and specific activity of [^{3}H]STX used in these experiments are well calibrated.

To examine the size of the recombinant saxiphilin proteins, 15 μL of culture medium from cells expressing R-sax and C-sax was subjected to SDS−PAGE and immunoblot

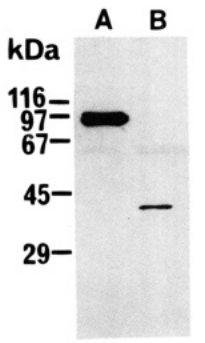

FIGURE 4: Immunoblot of cell culture medium from High Five cells infected with baculovirus expression vectors. An aliquot (15 μL) of medium from cells expressing R-sax (lane A) or C-sax (lane B) was subjected to SDS−PAGE and electroblotted onto a nitrocellulose membrane. The electroblotted membrane was exposed to anti-saxiphilin antibody and processed for chemiluminescence detection. Positions of standard molecular weight markers are shown at the left.

analysis. In this assay, affinity-purified polyclonal antibodies to native bullfrog saxiphilin (Li et al., 1993) were used to detect the recombinant forms of saxiphilin. This experiment resolved a single immunoreactive band in each of the two samples. As shown in Figure 4, culture medium from cells infected with baculovirus coding for R-sax exhibited a band with an apparent molecular mass of 92 kDa, and the corresponding sample for C-sax exhibited a band of 38 kDa, relative to protein molecular weight standards. These values are in close agreement to the theoretical protein molecular masses of R-sax (90.9 kDa) and C-sax (39.6 kDa), calculated from the respective primary sequences, assuming removal of the 19-residue, N-terminal signal sequence. To further characterize the recombinant R-sax protein, [^{3}H]STX binding activity was purified from High Five cell culture medium using a procedure similar to that described for the purification of native saxiphilin from bullfrog plasma (Li & Moczydlowski, 1991). The resulting preparation consisted of a predominant component with a molecular mass of 92 kDa corresponding to the band identified in the immunoblot of Figure 4 (not shown). When this sample was subjected to automated amino acid sequencing, a major sequence corresponding to the first 21 residues of native saxiphilin was evident, although there also appeared to be lower levels of an unrecognized sequence contaminating the sample. Despite the impurity, these data indicate that the native signal sequence of saxiphilin is correctly cleaved en route to secretion by the cultured insect cells.

Kinetics and pH Dependence of [^{3}H]STX Binding to R-sax and C-sax. To investigate the basis for the ~4-fold difference in the K_D of [^{3}H]STX for the whole recombinant saxiphilin protein vs that for the C-lobe, we measured the kinetics and pH dependence of [^{3}H]STX binding. Panels A and B of Figure 5 show the time course of association and dissociation, respectively, of [^{3}H]STX for both R-sax and C-sax in culture medium from infected insect cells. The time course of association measured in the presence of 10 nM [^{3}H]STX was virtually identical for the two proteins and was well described by an exponential function, as expected for pseudo-first-order kinetics. The derived bimolecular association rate constants for [^{3}H]STX binding were (1.8 ± 0.1) × 10^6 s^{-1} M^{-1} and (1.7 ± 0.1) × 10^6 s^{-1} M^{-1} for R-sax and C-sax, respectively. However, the time course of [^{3}H]-STX dissociation as measured by the rate of exchange with excess unlabeled STX was significantly faster for the C-sax

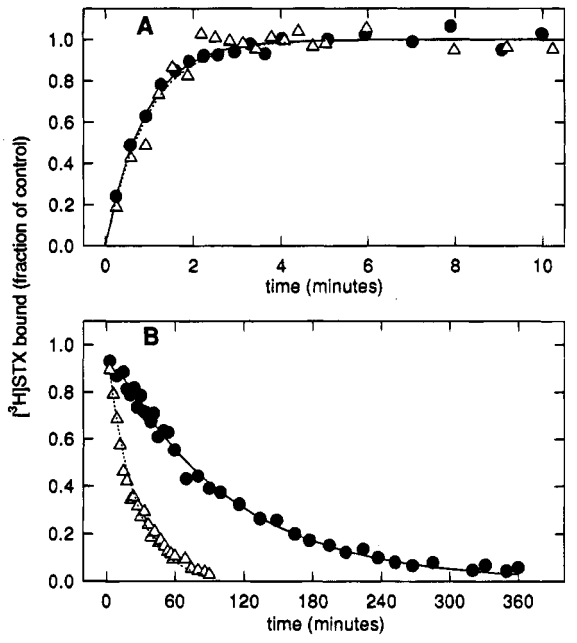

FIGURE 5: Kinetics of [^{3}H]STX binding to recombinant saxiphilin. (A) Association time course of [^{3}H]STX (10 nM) binding to an aliquot of medium from High Five cells expressing R-sax (●) or C-sax (△). Data points are normalized to the equilibrium level of binding and fit using a pseudo-first-order rate constant of 0.0187 s^{-1} (solid line, R-sax) or 0.0176 s^{-1} (dotted line, C-sax). (B) Time course of [^{3}H]STX dissociation from R-sax (●) and C-sax (△). Data points are normalized to [^{3}H]STX bound before addition of excess unlabeled STX and fit to a first-order rate constant of 1.64×10^{-4} s^{-1} (solid line, R-sax) and 7.02×10^{-4} s^{-1} (dotted line, C-sax).

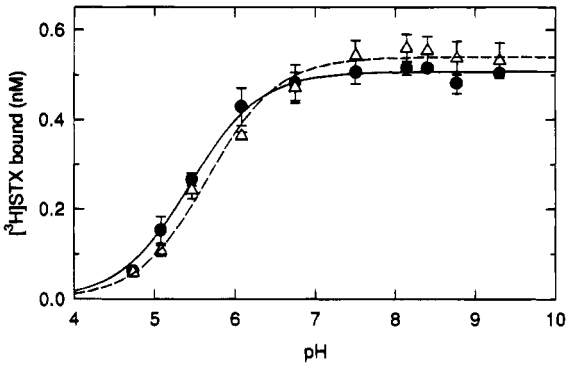

FIGURE 6: pH dependence of [^{3}H]STX binding to recombinant saxiphilin. An aliquot of medium from High Five cells expressing R-sax (●) or C-sax (△) was assayed for equilibrium binding in the presence of 5 nM [^{3}H]STX at various pH values. Data points are the mean of duplicate samples and are expressed as nanomolar bound [^{3}H]STX corrected for nonspecific binding in the presence of 10 μM STX. Data are fit to the equation $y = B_{max}\,K_{0.5}/(K_{0.5} + [H^+])$, using $pK_{0.5} = 5.44$ and $B_{max} = 0.51$ nM (R-sax, solid line) or $pK_{0.5} = 5.66$ and $B_{max} = 0.54$ nM (C-sax, dotted line).

protein compared to R-sax (Figure 5B). In both cases the time course was well described by a single-exponential decay. The best-fit values of the corresponding dissociation rate constants were $(1.64 \pm 0.03) \times 10^{-4}$ s^{-1} for R-sax and $(7.02 \pm 0.16) \times 10^{-4}$ s^{-1} for C-sax. Figure 6 shows the pH dependence of equilibrium binding of [^{3}H]STX to R-sax and C-sax. The data conform to a one-site H^+ titration curve as previously described for native saxiphilin (Llewellyn & Moczydlowski, 1994). The $pK_{0.5}$ values derived for the two proteins were very similar, 5.44 ± 0.04 and 5.66 ± 0.05 for R-sax and C-sax, respectively, as compared to $pK_{0.5} = 5.70$ for native saxiphilin assayed under the same conditions.

DISCUSSION

In this study, baculovirus-mediated expression was used to successfully produce recombinant saxiphilin and a deletion mutant of saxiphilin consisting of the predicted C-lobe domain. To our knowledge, this is the first report of expression of a vertebrate relative of the transferrin family in insect cells. It was expected that invertebrate cells would be suitable for expression of saxiphilin since endogenous Fe^{3+}-binding transferrin proteins have been previously identified in insects (Bartfeld & Law, 1990; Jamroz et al., 1993; Kurama et al., 1995). Recombinant human serum transferrin and human lactoferrin have been expressed in the mammalian BHK cell line (baby hamster kidney cells) using the pNUT expression vector (Mason et al., 1993; Stowell et al., 1991). This latter system was also used to express functionally active forms of the half-molecule N-lobe of both human transferrin and human lactoferrin (Funk et al., 1990; Mason et al., 1991; Day et al., 1992). Previous attempts to express transferrin in procaryotic cells such as *E. coli* have not yielded functionally active protein with Fe binding activity, presumably due to improper folding (Funk et al., 1990; Ikeda et al., 1992). The protein folding and/or secretion apparatus of eucaryotic cells appears to be necessary for functional expression of secreted forms of whole and N-lobe and C-lobe domains of transferrin, lactoferrin, and their relatives such as saxiphilin.

Equilibrium binding analysis (Figure 2) indicates that recombinant saxiphilin (R-sax) binds [^{3}H]STX with virtually the same affinity ($K_D \simeq 0.2$ nM) as native saxiphilin from bullfrog plasma (Llewellyn & Moczydlowski, 1994). Native and recombinant saxiphilin appear to have the same molecular mass (~91 kDa) as judged by virtual comigration of the respective bands on SDS−PAGE (data not shown). The kinetics and pH dependence of [^{3}H]STX binding to R-sax are also very similar to that described for native saxiphilin (Llewellyn & Moczydlowski, 1994). At pH 7.4 and 0 °C, the observed bimolecular association rate constants are 8.0×10^5 s^{-1} M^{-1} for native saxiphilin and 18.0×10^5 s^{-1} M^{-1} for R-sax, whereas the measured dissociation rate constants are 1.44×10^{-4} s^{-1} and 1.64×10^{-4} s^{-1}, respectively. Thus, by the criteria of molecular size and ligand-binding properties, recombinant saxiphilin produced in insect cells so closely resembles the native bullfrog protein that it should be useful for the further analysis of its structure and cellular function.

It was previously deduced that the native 91-kDa saxiphilin protein contains one high-affinity binding site for [^{3}H]STX (Llewellyn & Moczydlowski, 1994). The new finding of [^{3}H]STX binding by the ~40-kDa C-terminal domain, C-sax, establishes that the STX binding site is located in this portion of the protein. The similar pH dependence of [^{3}H]STX binding to C-sax and R-sax (Figure 6) further implies that the protonatable protein residue responsible for modulating the kinetics of ligand binding is also located in the C-lobe. Since there is one mutation (corresponding to Ser-616 to Pro in native saxiphilin) in the expressed form of C-sax that was inadvertently introduced by PCR, we cannot unambiguously attribute the slightly lower [^{3}H]STX binding affinity and faster dissociation rate of C-sax to the loss of interlobe interactions that may result from deletion of the N-lobe of saxiphilin. On the basis of sequence alignment to lactoferrin, this mutated residue is predicted to lie with a short 3_{10} turn

(Anderson et al., 1989). Since this turn lies on the exterior surface of lactoferrin distant from the Fe binding site in the C-lobe, it seems likely that this mutation would not have a major impact on the structure of the [^{3}H]STX binding site that we hypothesize to be located within the interdomain cleft between subdomains C1 and C2. However, this proline residue may partially disrupt local secondary structure and be the basis for the observed 4-fold faster rate of [^{3}H]STX dissociation from C-sax vs R-sax. With the baculovirus expression system described here, the effect of altering this residue and other residues on the kinetics of STX binding can now be addressed by site-specific mutagenesis.

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REFERENCES

Anderson, B. F., Baker, H. M., Norris, G. E., Rice, D. W., & Baker, E. N. (1989) *J. Mol. Biol. 209*, 711−734.

Bailey, S., Evans, R. W., Garratt, R. C., Gorinsky, B., Hasnain, S., Horsburgh, C., Jhoti, H., Lindley, P. F., Mydin, A., Sarra, R., & Watson, J. L. (1988) *Biochemistry 27*, 5804−5812.

Baker, E. N., Baker, H. M., Smith, C. A., Stebbins, M. R., Kahn, M., Hellström, K. E., & Hellström, I. (1992) *FEBS Lett. 298*, 215−218.

Bartfeld, N. S., & Law, J. H. (1990) *J. Biol. Chem. 265*, 21684−21691.

Bullen, J. J. (1987) in *Iron and Infection: Molecular, Physiological and Clinical Aspects* (Bullen, J. J., & Griffits, E., Eds.) pp 211−241, John Wiley & Sons, New York.

Crichton, R. R. (1991) *Inorganic Biochemistry of Iron Metabolism*, pp 1−263, Horwood, New York.

Dautry-Varsat, A. (1986) *Biochimie 68*, 375−381.

Day, C. L., Stowell, K. M., Baker, E. N., & Tweedie, J. W. (1992) *J. Biol. Chem. 267*, 13857−13862.

Doyle, D. D., Wong, M., Tanaka, J., & Barr, L. (1982) *Science 215*, 1117−1119.

Ellis, K. J., & Morrison, J. F. (1982) *Methods Enzymol. 87*, 405−426.

Funk, W. D., MacGillivray, R. T. A., Mason, A. B., Brown, S. A., & Woodworth, R. C. (1990) *Biochemistry 29*, 1654−1660.

Gerstein, M., Anderson, B. F., Norris, G. E., Baker, E. N., Lesk, A. M., & Chothia, C. (1993) *J. Mol. Biol. 234*, 357−372.

Green, B. N., MacGillivray, R. T. A., & Woodworth, R. C. (1993) *Biochemistry 32*, 5472−5479.

Griffiths, E., & Bullen, J. J. (1987) in *Iron and Infection: Molecular, Physiological and Clinical Aspects* (Bullen, J. J., & Griffits, E., Eds.) pp 171−209, John Wiley & Sons, New York.

He, J., & Furmanski, P. (1995) *Nature 373*, 721−724.

Ikeda, R. A., Bowman, B. H., Yang, F., & Lokey, L. K. (1992) *Gene 117*, 265−269.

Jamroz, R. C., Gasdaska, J. R., Bradfield, J. Y., & Law, J. H. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 1320−1324.

Kurama, T., Kurata, S., & Natori, S. (1995) *Eur. J. Biochem. 228*, 229−235.

Laemmli, U. K. (1970) *Nature 227*, 680−685.

Li, Y., & Moczydlowski, E. (1991) *J. Biol. Chem. 266*, 15481−15487.

Li, Y., Llewellyn, L., & Moczydlowski, E. (1993) *Mol. Pharmacol. 44*, 742−748.

Llewellyn, L., & Moczydlowski, E. G. (1994) *Biochemistry 33*, 12312−12322.

Mahar, J., Lukacs, G. L., Li, Y., Hall, S., & Moczydlowski, E. (1991) *Toxicon 29*, 53−71.

Malthiery, Y., & Lissitzky, S. (1987) *Eur. J. Biochem. 165*, 491−498.

Mason, A. B., Funk, W. D., MacGillivray, R. T. A., & Woodworth, R. C. (1991) *Protein Expression Purif. 2*, 214−220.

Mason, A. B., Miller, M. K., Funk, W. D., Banfield, D. K., Savage, K. J., Oliver, R. W. A., Green, B. N., MacGillivray, R. T. A., & Woodworth, R. C. (1993) *Biochemistry 32*, 5472−5479.

Morabito, M. A., & Moczydlowski, E. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 2478−2482.

Morabito, M. A., & Moczydlowski, E. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 6651.

Morabito, M., Llewellyn, L. E., & Moczydlowski, E. (1995) *Biophys. J. 68*, A405.

O'Reilly, D. R., Miller, L. K., & Luckow, V. A. (1994) *Baculovirus Expression Vectors: A Laboratory Manual*, pp 1−347, Oxford University Press, New York.

Richardson, D., & Baker, E. (1992) *J. Biol. Chem. 267*, 13972−13979.

Rose, T. M., Plowman, G. D., Teplow, D. B., Dreyer, W. J., Hellström, K. E., & Brown, J. P. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1261−1265.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.

Stowell, K. M., Rado, T. A., Funk, W. D., & Tweedie, J. W. (1991) *Biochem. J. 276*, 349−355.

Summers, M. D., & Smith, G. E. (1987) *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures*, pp 1−56, Texas Agricultural Experiment Station, College Station, TX.

Thorstensen, K., & Romslo, I. (1990) *Biochem. J. 271*, 1−10.

Welch, S. (1992) *Transferrin: The Iron Carrier*, pp 1−291, CRC Press, Boca Raton, FL.

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