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Biochemical and Immunochemical Comparison of Saxiphilin and Transferrin, Two Structurally Related Plasma Proteins from *Rana catesbeiana*

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SUMMARY

Saxiphilin is a ~90-kDa protein in bullfrog plasma that binds the neurotoxin saxitoxin (STX) with high affinity (K_d, ~0.2 nM). The relationship between saxiphilin and transferrin was examined because partial sequencing of saxiphilin previously revealed an unexpected homology to members of the transferrin family of Fe^{3+}-binding proteins. Transferrin was purified from bullfrog plasma and shown to be distinct from saxiphilin on the basis of its size (~78 kDa), chromatographic behavior, visible absorption spectrum, and ligand-binding properties. High affinity binding of [^{3}H]STX was found to be a distinctive property of saxiphilin that was not exhibited by transferrins from various species of animals. Conversely, under conditions appropriate for transferrins, purified saxiphilin did not bind $^{55}Fe^{3+}$, implying that it is not involved in iron metabolism. Polyclonal antibodies raised against native saxiphilin precipitated [^{3}H]STX-binding activity from whole bullfrog plasma. On immunoblots such antibodies recognized the denatured saxiphilin protein but only weakly labeled bullfrog transferrin. In an enzyme-linked immunosorbent assay using native proteins, antisaxiphilin antibodies weakly cross-reacted with transferrin from bullfrog and a number of other species. Likewise, antibodies against human transferrin cross-reacted with saxiphilin in a similar immunosorbent assay. These results lead to the conclusion that saxiphilin is not bullfrog transferrin but is structurally related to the transferrin family. As a novel member of the transferrin superfamily, saxiphilin may help to uncover new functions mediated by this class of proteins.

STX, a small heterocyclic guanidinium compound, is a potent neurotoxin that is produced by certain dinoflagellates and cyanobacteria (1). A well known aspect of the biology of STX is the widespread distribution of this toxin in various marine animals. In toxicology, STX is associated with the problem of "paralytic shellfish poisoning" that sporadically occurs in conjunction with plankton blooms (2). Paralysis induced by STX poisoning is due to blockage of voltage-dependent Na^+ channels of electrically excitable cells at an external site associated with the conducting pore (3). In addition to STX-sensitive Na^+ channels, tissues from various amphibians and reptiles have been found to contain a different soluble protein, named saxiphilin, that specifically binds STX with high affinity (4, 5). At present, it is unknown whether STX binding to saxiphilin has any physiological significance, but such a protein might have useful pharmacological applications in reversal of STX block of excitable cells and/or in antidote therapy.

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Partial sequencing of saxiphilin purified from plasma of the North American bullfrog (*Rana catesbeiana*) revealed that fragments of this 90-kDa protein exhibit considerable sequence homology (40–70% identity) to vertebrate transferrins (6). Transferrins are a family of monomeric Fe^{3+}-binding glycoproteins of M_r ~80,000, including serum transferrin, lactoferrin, melanotransferrin, and ovotransferrin (7, 8). Some of these proteins exhibit antimicrobial activity due to their high affinity for Fe^{3+} (K_d, ~10^{-20} M); however, the essential role of serum transferrin is to supply eukaryotic cells with Fe^{3+}, which is necessary for growth as a requisite cofactor of numerous metalloproteins. Transferrins contain two internally homologous domains that each bind one Fe^{3+} ion and one bicarbonate anion, except for human melanotransferrin (9, 10) and a transferrin isolated from the tobacco hornworm *Manduca sexta* (11), which both appear to have only one functional Fe^{3+}-binding domain.

Because bullfrog transferrin has not yet been sequenced or cloned, the unexpected homology between saxiphilin and members of the transferrin family raised the possibility that saxiphilin is an unusual derivative or isoform of transferrin itself.

ABBREVIATIONS: STX, saxitoxin; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NTA, nitrilotriacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

To address this question, we have separately purified saxiphilin and transferrin from bullfrog plasma and compared the size, ligand-binding properties, and immunochemical cross-reactivity of these two proteins. The results indicate that saxiphilin is a distinct protein that does not bind Fe^{3+} but nevertheless contains related antigenic determinants that reflect underlying structural similarity to transferrins.

Experimental Procedures

Materials. Various materials and animals used in this study were obtained from the following commercial sources: [^{3}H]STX (20–40 Ci/mmol) and $^{55}FeCl_3$ (70 Ci/mol) from Amersham; STX and Pansorbin (*Staphylococcus aureus* cells) from Calbiochem; Biogel P6 from Bio-Rad; CNBr-activated Sepharose 4B, S-Sepharose, Sephadex G-200, and DEAE-Sephadex A-50 from Pharmacia; NTA, apotransferrin from human, horse, cow, and rabbit, transferrin from mouse and guinea pig, human lactoferrin, chicken ovotransferrin, and goat antiserum against human transferrin from Sigma; peroxidase-conjugated goat IgG against rabbit immunoglobulins and peroxidase-conjugated rabbit IgG against goat IgG from Cappel (Organon Teknika Corp.); and adult bullfrogs (*Rana catesbeiana*) from Connecticut Valley Biological Supply.

Purification of saxiphilin and preparation of antisaxiphilin antibodies. Saxiphilin, assayed by [^{3}H]STX binding, was purified to homogeneity from bullfrog plasma by column chromatography on heparin-Sepharose and chromatofocusing as described previously (6). Polyclonal antiserum to native saxiphilin was raised in rabbits by Pocono Rabbit Farm and Laboratory (Canadensis, PA). Antigen injections of pure saxiphilin followed a schedule of 200 μg of protein in Freund's complete adjuvant injected intradermally on the first day, 100 μg in Freund's incomplete adjuvant injected intradermally on day 14, and 25-μg intramuscular boost injections in incomplete adjuvant on day 28 and every 4 weeks thereafter. A high titer of antisaxiphilin antibodies was observed after 5 weeks with the enzyme-linked immunosorbent assay described below. Antiserum (~15 ml) was collected from weekly bleeds. Antisera obtained from two different rabbits exhibited similar levels of reactivity.

Antisaxiphilin antibodies used in this work were affinity-purified using saxiphilin as the ligand. Pure saxiphilin (0.5 mg) was covalently coupled to 0.5 ml of swelled CNBr-activated Sepharose 4B according to the manufacturer's instructions. The prepared column was equilibrated with bicarbonate buffer (100 mM $NaHCO_3$, 500 mM NaCl, pH 8.3), and 0.1 ml of rabbit antisaxiphilin antiserum diluted to 1 ml in bicarbonate buffer plus 0.5% Tween 20 detergent was applied and recycled three times through the column. The column was then washed with 10 ml of bicarbonate buffer and eluted with 5 ml of 100 mM glycine, pH 2.8. A small peak of specific antibodies that eluted after the pH step was neutralized to pH 7.5 with Tris base, stored frozen, and used in various immunoassays.

Purification of bullfrog transferrin. A sample (10 ml) of previously collected bullfrog plasma was thawed and supplemented with 10 mM $NaHCO_3$ and 15 μM $Fe(NTA)_2$ premixed with 45 μCi of $^{55}FeCl_3$. This ^{55}Fe-labeled plasma sample was first subjected to gel filtration chromatography on a 2.5- × 50-cm column of Sephadex G-200 equilibrated with 100 mM Tris·HCl, 1 M NaCl, pH 7.8, and was eluted at 12 ml/hr with the same buffer. A peak of soluble, protein-bound ^{55}Fe eluting after the void volume was pooled and dialyzed against 3 liters of 20 mM Tris·HCl, pH 7.8. This sample was applied to a 2.5- × 25-cm column of DEAE-Sephadex A-50 equilibrated with 20 mM Tris·HCl, pH 7.8, was eluted at 12 ml/hr with a linear gradient of 400 ml of 20–500 mM Tris·HCl, pH 7.8, and was collected in 12.5-ml fractions. Aliquots (10 μl) of various fractions were assayed for ^{55}Fe by liquid scintillation counting. Specific binding of [^{3}H]STX was assayed on 10-μl fraction aliquots as described (6). Protein was monitored by measuring absorbance at 280 nm, and the salt gradient was monitored by measuring conductivity of 1/100 dilutions of various fractions. A single major peak (Fig. 1) of ^{55}Fe was identified as bullfrog transferrin by its

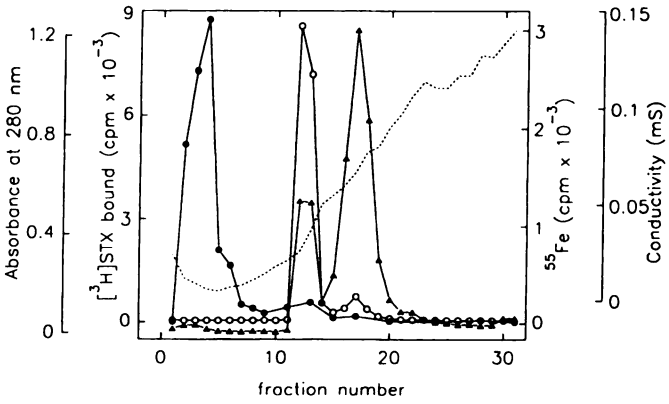

Fig. 1. Separation of [^{3}H]STX- and ^{55}Fe-binding activities in bullfrog plasma. A 10-ml sample of bullfrog plasma was labeled with $^{55}Fe(NTA)_2$, partially purified by gel filtration on a column of Sephadex G-200, and subjected to anion exchange chromatography on a column of DEAE-Sephadex A-50, as described in Experimental Procedures. Various fractions from the DEAE-Sephadex A-50 column were assayed for protein (▲), specific binding of [^{3}H]STX (●), ^{55}Fe (○), and conductivity (– – –). Similar results were obtained in three different experiments.

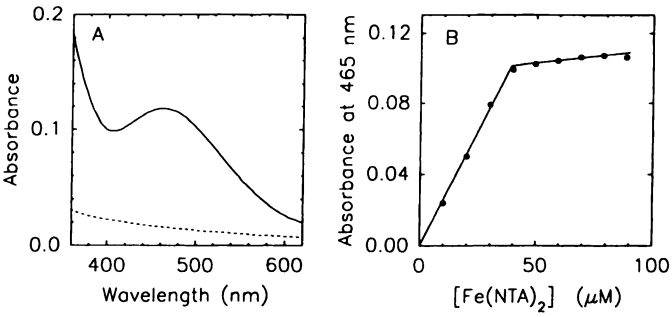

Fig. 2. Visible absorption spectrum and Fe^{3+} titration of bullfrog apotransferrin. A, Spectrum of 1.6 mg/ml apotransferrin before (– – –) and after (——) addition of 50 μM $Fe(NTA)_2$. B, Absorbance change at 465 nm measured after addition of the indicated concentrations of $Fe(NTA)_2$ to 1.6 mg/ml apotransferrin.

characteristic orange-pink color and visible absorption spectrum (Fig. 2A) with a maximum at 465 nm.

Iron was removed from purified transferrin by addition of 1 mM NTA and 2 mM EDTA, adjustment to pH 4.2 with acetic acid, and incubation overnight at 0°. The sample was then dialyzed against 1 mM MOPS-NaOH, 100 mM NaCl, pH 7.4, and lyophilized to 1 ml. Residual chelating agent was removed by gel filtration on a 15-ml column of Biogel P6 eluted with 100 mM NaCl, 1 mM MOPS-NaOH, pH 7.4. The yield of apotransferrin was ~8 mg.

Because [^{3}H]STX binding experiments indicated that bullfrog transferrin purified by the method described above contained a trace amount of contaminating saxiphilin, an additional purification step was undertaken for use in immunochemical assays. S-Sepharose was used because saxiphilin is a basic protein and was previously found to adsorb to this medium (5). A column (4 ml) of S-Sepharose was equilibrated with 25 mM sodium acetate, 10 mM MES-NaOH, pH 6.0. Bullfrog apotransferrin (320 μg) diluted to 1 ml with equilibration buffer was applied to the column at 15 ml/hr. The column was eluted with 24 ml of 100 mM sodium acetate and 12 ml of 150 mM sodium acetate in 10 mM MES-NaOH, pH 6.0. The peak of protein eluting after the final step of 150 mM sodium acetate was pooled, dialyzed against 10 mM NaCl, 1 mM HEPES-NaOH, pH 7.4, and concentrated by lyophilization.

Fe^{3+} titration of apotransferrin and absorption spectrum. Stock solutions of 10 mM $Fe(NTA)_2$ were prepared fresh by dissolving $FeCl_3 \cdot 6H_20$ in an acidic solution of NTA at a ratio of 2.2 NTA/Fe and adjusting the pH to 4.0 with NaOH. Fe^{3+} binding to apotransferrin was monitored by measuring absorbance at 465 nm with a Perkin-Elmer

λ9 UV-visible scanning spectrophotometer. Absorbance was recorded 10 min after addition, with mixing, of consecutive 2-μl aliquots of 5 mM $Fe(NTA)_2$ to a quartz cuvette (1-cm path length) containing 1.0 ml of 1.6 mg/ml apotransferrin in 20 mM HEPES-NaOH, 20 mM $NaHCO_3$, 100 mM NaCl, pH 7.4. At the equivalence point, the spectrum of Fe_2-transferrin was recorded against a reference cuvette containing titration buffer minus protein. To determine the stoichiometry of Fe^{3+} binding, the transferrin protein concentration was based on quantitative amino acid analysis performed by the Yale Protein Chemistry Facility. The amino acid composition of bullfrog transferrin was similar to that reported previously (12). Protein determined by this method was corrected for proline, cysteine, and tryptophan, which were not measured directly but were assumed to be equal to 10.7 weight percent, based on the known composition of transferrin cloned from *Xenopus laevis* (13).

Immunoprecipitation of [^{3}H]STX-binding activity. A sample of 35 μg of bullfrog plasma was incubated with 11 nM [^{3}H]STX, 10 mM MOPS-NaOH, 200 mM choline chloride, pH 7.4, and various amounts (0.2–25 μg) of affinity-purified antisaxiphilin antibodies were added in a final volume of 0.5 ml. Control reactions included 40 μM STX or preimmune rabbit serum instead of specific antibodies. Samples were incubated for 1 hr on ice, and 50 μl of Pansorbin (10%, w/v, *S. aureus* cells) were added for an additional 1-hr incubation. The samples were centrifuged at 12,000 × *g* for 3 min. The pellets were washed three times in incubation buffer and counted in a scintillation counter.

Immunoblots. Samples of 1–10 μg of bullfrog plasma, purified saxiphilin, and transferrin were subjected to SDS-PAGE (14) using 7.5% polyacrylamide gels and were electroblotted onto nitrocellulose membranes (Gelman Biotrace NT) using a Bio-Rad Trans-Blot apparatus, as described (6). The membrane blots were probed with affinity-purified antisaxiphilin antibody (1/1500 dilution of 0.25 mg/ml antibody) and developed according to instructions for the Western blot analysis system (Amersham), which uses a peroxidase-conjugated anti-rabbit antibody and a chemiluminescence reaction to expose film.

Enzyme-linked immunosorbent assay. Purified saxiphilin or transferrin from various sources was diluted to 5 μg/ml in PBS (10 mM NaH_2PO_4, 150 mM NaCl, pH 7.2), and 50 μl were added to individual wells of a polystyrene microtiter plate (Corning 25860). The plates were incubated at 4° overnight for adsorption of antigens. After the plate was drained, each well was blocked with 150 μl of 4% nonfat dry milk in PBS and incubated for 1 hr at room temperature. The plates were drained and 50 μl of various serial dilutions (in 4% milk) of rabbit antisaxiphilin antibody or goat anti-human transferrin antiserum were added to each well. After a 1-hr incubation, the drained plate was washed three times with 150 μl/well of 4% milk. Each well then received 150 μl of a 1/500 dilution of peroxidase-conjugated second antibody. After a 1-hr incubation, wells were washed three times with 150 μl of 4% milk and then two times with PBS. This was followed by addition of 75 μl/well of substrate solution (0.4% *o*-phenylenediamine, 0.0126% H_2O_2, in PBS). After a 30-min incubation, 50 μl of 5 N H_2SO_4 were added to stop the reaction and absorbance at 490 nm was read with a microtiter plate spectrophotometer. The same protocol was followed for experiments with increasing antigen concentrations at fixed antibody dilutions of 1/2000.

Results

Chromatographic separation of [^{3}H]STX-binding activity and $^{55}Fe^{3+}$-binding activity. $^{55}Fe^{3+}$ and [^{3}H]STX were used to monitor binding activity of these ligands during chromatographic separation of transferrin from saxiphilin in bullfrog plasma. Plasma transferrin was prelabeled with a complex of $^{55}Fe^{3+}$ and NTA, in the presence of 10 mM bicarbonate. NTA serves to provide a soluble form of chelated Fe^{3+} that can readily bind to apotransferrin (7). Transferrin was purified from ^{55}Fe-labeled plasma by gel filtration on Sephadex G-200, followed by anion exchange chromatography on DEAE-Sephadex. Assays of fractions from the Sephadex G-200 column revealed co-migration of [^{3}H]STX-binding activity and protein-bound ^{55}Fe after the void volume (data not shown). This initial co-migration of the two binding activities on a gel filtration column is consistent with the similar elution volumes of saxiphilin activity and human transferrin previously observed using high performance size exclusion chromatography (5). Further fractionation on DEAE-Sephadex of the Sephadex G-200 pool containing protein-bound ^{55}Fe resolved saxiphilin and transferrin activity into two distinct peaks (Fig. 1). Consistent with the high isoelectric point (pI ~10.9) of saxiphilin, as determined by isoelectric focusing (5), [^{3}H]STX-binding activity readily passed through the DEAE-Sephadex column at pH 7.8 and low ionic strength. In contrast, a single major peak corresponding to bullfrog transferrin eluted from this column at higher ionic strength, as identified by a characteristic orange-pink color of the fractions containing ^{55}Fe.

After removal of Fe^{3+} from purified bullfrog transferrin by prolonged incubation at pH 4.2 in the presence of 1 mM NTA and 2 mM EDTA, the protein was further characterized by spectrophotometric titration of absorbance at 465 nm with $Fe(NTA)_2$. This titration (Fig. 2B) exhibited a sharp equivalence point characteristic of other transferrins and a slope of 2590 M^{-1} cm^{-1}/Fe^{3+} site, which is similar to reported values for the extinction coefficient of human transferrin (ϵ_{465} = 2500–2600 M^{-1} cm^{-1}) (15, 16). An Fe^{3+}-binding capacity of 1.9 mol of Fe^{3+}/mol of bullfrog transferrin was calculated by dividing the observed $Fe(NTA)_2$ equivalence point by the protein concentration, as determined by quantitative amino acid analysis, and assuming a protein molecular weight (M_r = 77,640) equal to that of transferrin from the African clawed frog *Xenopus laevis* (13). This Fe^{3+}-binding capacity is consistent with two functional Fe^{3+} sites/transferrin molecule, as found for all known serum transferrins from other vertebrates (7). The absorption spectrum of bullfrog transferrin (Fig. 2A) exhibited an Fe^{3+}-dependent maximum in the visible region at 465 nm and a minimum near 405 nm, which is typical of transferrins from various sources (7). The spectrum of Fe^{3+}-saturated bullfrog transferrin was also characterized by absorbance ratios of A_{465}/A_{280} = 0.046 and A_{465}/A_{410} = 1.18. The former ratio is similar to that of native human Fe_2-transferrin (A_{465}/A_{280} = 0.046) (17) and the recombinant form of the amino-terminal half-molecule of human Fe-transferrin (A_{465}/A_{280} = 0.048) (18). However, the A_{465}/A_{410} ratio of bullfrog transferrin is somewhat lower than that of the native and recombinant forms of human transferrin (A_{465}/A_{410} = 1.34–1.41) (16, 18, 19). The absence of an absorbance peak at 410 nm implies that the preparation is not contaminated by heme, as also noted in other preparations of bullfrog transferrin (20, 21).

Demonstration of different molecular weights of purified saxiphilin and transferrin by SDS-PAGE. A comparative analysis of purified saxiphilin and transferrin preparations by SDS-PAGE is shown in Fig. 3. Purified transferrin exhibited a single predominant band migrating with an apparent molecular weight of 78,000 ± 1,000. The transferrin band was at the same position as one of the major protein bands of whole plasma, consistent with a typical serum protein content of ~10% transferrin (12). Saxiphilin was separately purified from bullfrog plasma by a procedure involving chromatofocusing, as described previously (6). Saxiphilin migrated on SDS-PAGE with a distinctly higher apparent molecular weight of

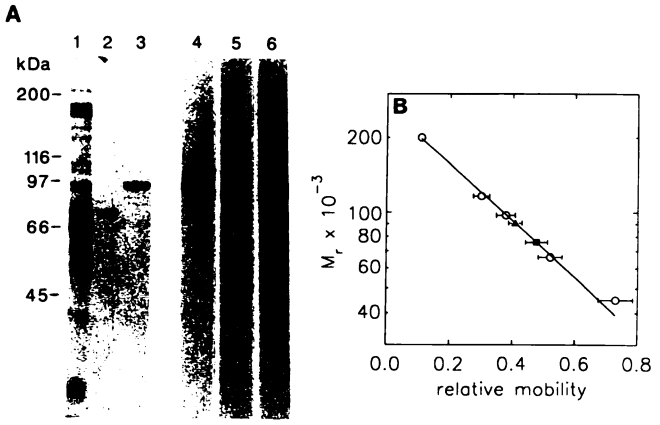

Fig. 3. SDS-PAGE and immunoblots of whole plasma, transferrin, and saxiphilin. A, *Lanes 1*, 2, and *3*, SDS-PAGE of 10 μg of bullfrog plasma, 1 μg of bullfrog transferrin, and 1 μg of bullfrog saxiphilin, respectively, stained with Coomassie blue. *Lanes 4*, *5*, and *6*, immunoblots of a duplicate of *lanes 1*, *2*, and *3*, respectively, probed with rabbit antisaxiphilin antibodies detected by peroxidase-conjugated anti-rabbit antibody and Amersham Enhanced Chemiluminescence reagents. B, Compilation of data from five SDS-PAGE experiments showing the relative mobility of bullfrog transferrin (■) and saxiphilin (▲) with respect to five molecular weight markers (○), i.e., myosin (M_r 200,000), β-galactosidase (M_r 116,000), phosphorylase *b* (M_r 97,400), bovine serum albumin (M_r 66,200), and hen ovalbumin (M_r 45,000).

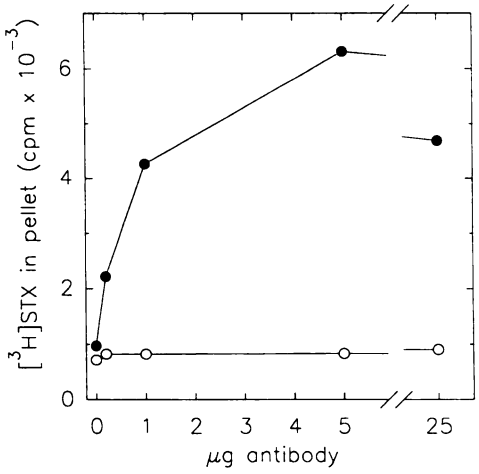

Fig. 4. Immunoprecipitation of [^{3}H]STX-binding activity from bullfrog plasma by antisaxiphilin antibodies. Bullfrog plasma (35 μg) was incubated with 11 nM [^{3}H]STX in the absence (●) or presence (○) of unlabeled STX and was subjected to precipitation with increasing amounts of antisaxiphilin antibody, as described in Experimental Procedures. *Data points* are the means of duplicate determinations. Similar results were obtained in three experiments.

90,000 ± 3,000 (Fig. 3B). The saxiphilin band exhibited a positive reaction when stained for carbohydrate by the periodic acid Schiff method (22) (data not shown). This indicates that, like transferrin, saxiphilin is a glycoprotein.

Discrimination of saxiphilin and transferrin by antisaxiphilin antibodies on immunoblots. Antiserum to native saxiphilin was raised in rabbits and polyclonal antibodies were affinity-purified on a column of saxiphilin coupled covalently to Sepharose 4B. Such antibodies were capable of immunoprecipitating [^{3}H]STX-binding activity from samples of crude bullfrog plasma (Fig. 4). This precipitation was dependent on the amount of antibody added, was abolished by an excess of unlabeled STX, and did not occur with preimmune rabbit serum. Samples of bullfrog plasma, transferrin, and saxiphilin subjected to SDS-PAGE were electroblotted onto nitrocellulose membranes. The resulting protein blots were probed with antisaxiphilin antibodies and developed by a chemiluminescence-based detection technique. Typical results in Fig. 3A show a strong reaction with pure saxiphilin and specific detection of an equivalent band in the sample corresponding to crude plasma. In contrast, the band corresponding to pure bullfrog transferrin exhibited weak reactivity in this assay. Control experiments using rabbit serum collected before immunization with saxiphilin showed no reaction (data not shown). The results of Figs. 3 and 4 demonstrate that the 90-kDa [^{3}H]STX-binding protein previously characterized as saxiphilin (6) is distinct from bullfrog serum transferrin, on the basis of size and reactivity to antisaxiphilin antibodies.

Evidence that saxiphilin and transferrin have different ligand-binding properties. Purified saxiphilin and bullfrog apotransferrin were compared in sensitive binding assays for $^{55}Fe^{3+}$ and [^{3}H]STX. In case the ability of saxiphilin to bind iron was masked under the conditions of the experiment with whole plasma in Fig. 1, we separately incubated pure saxiphilin and transferrin (as a control) with excess $^{55}Fe(NTA)_2$ and 10 mM $NaHCO_3$ at pH 5. After 12 hr of incubation, this mixture was adjusted to pH 7 and protein-bound $^{55}Fe^{3+}$ was separated from free $^{55}Fe(NTA)_2$ on a size exclusion column (Biogel P6). This technique allowed us to readily measure binding of $^{55}Fe^{3+}$ to bullfrog apotransferrin but showed no detectable binding of $^{55}Fe^{3+}$ to saxiphilin (data not shown). Correspondingly, when transferrins from various species were tested in comparison with saxiphilin for specific binding of 5 nM [^{3}H]STX, none of the nonamphibian transferrin proteins displayed significant binding of this neurotoxin (Table 1). In this experiment, 2 μg of various transferrins were tested versus 0.0074 μg of saxiphilin, to enhance detection of possible low affinity binding of [^{3}H]STX by transferrins. Bullfrog transferrin purified by chromatography on DEAE-Sephadex did exhibit a small amount of [^{3}H]STX binding, but the low specific activity of this binding (~12 pmol/mg) (Table 1) suggested that it could be due to trace contamination (~0.1%) by saxiphilin. This residual [^{3}H]STX binding was greatly reduced by subjecting bullfrog transferrin

TABLE 1

Lack of [^{3}H]STX binding by various transferrins

Samples of various transferrins (2 μg) or saxiphilin (0.0074 μg) were incubated in 100 μl of 100 mM MOPS-NaOH, pH 7.4, 100 mM NaCl, 10 mM $NaHCO_3$, 5 nM [^{3}H]STX, in the absence or presence of 10 μM unlabeled STX. After 1 hr at 0°, bound [^{3}H]STX was separated from free ligand as described (6). Values of cpm were converted to pmol bound using the measured specific activity of [^{3}H]STX (13,980 cpm/pmol). Specific binding is reported as the difference between assays in the absence and presence of 10 μM STX. Results are expressed as specific activity (pmol bound per mg of the tested protein), and reported values are the mean ± standard deviation of four measurements.

Sample	[^{3}H]STX bound
	pmol/mg
Human apotransferrin	0.2 ± 0.1
Human lactoferrin	0.0 ± 0.4
Bovine apotransferrin	−0.1 ± 0.6
Horse apotransferrin	−0.1 ± 0.3
Rabbit apotransferrin	0.3 ± 0.3
Guinea pig transferrin	−0.1 ± 0.3
Mouse transferrin	0.0 ± 0.1
Chicken ovotransferrin	0.1 ± 0.2
Bullfrog apotransferrin (before S-Sepharose)	12 ± 1.1
Bullfrog apotransferrin (after S-Sepharose)	1.7 ± 0.1
Bullfrog saxiphilin	12,000 ± 1,200

to an additional step of chromatography on S-Sepharose (Table 1), a medium that was previously found to effectively adsorb saxiphilin (5). The measured specific activity of [^{3}H]STX binding to pure saxiphilin (12,000 pmol/mg) is approximately equivalent to a 1:1 stoichiometry for a 90-kDa protein. A more rigorous Scatchard analysis of [^{3}H]STX binding to pure saxiphilin has confirmed this 1:1 binding stoichiometry.[1] Thus, our results indicate that saxiphilin binds 1 mol of [^{3}H]STX/mol of protein, whereas bullfrog transferrin binds 2 mol of Fe^{3+}/mol of protein.

Evidence of immunological cross-reactivity between saxiphilin and various transferrins. The antigenic relationship between saxiphilin and bullfrog transferrin was explored further by examining the reactivity of affinity-purified antisaxiphilin antibodies in an enzyme-linked immunosorbent assay. Fig. 5 shows the results of an experiment in which fixed amounts (250 ng) of pure saxiphilin and bullfrog transferrin were incubated with serial dilutions of antibody. As expected from the results of immunoblot analysis (Fig. 3A), the antisaxiphilin antibody was strongly reactive with saxiphilin, but a weak reaction with bullfrog transferrin was also detected at high antibody concentration. As noted above, [^{3}H]STX binding measurements suggested that nominally pure preparations of bullfrog transferrin may contain trace amounts of saxiphilin. Because such contamination could affect the interpretation of the immunosorbent assay results, we compared bullfrog transferrin before and after additional purification by S-Sepharose chromatography. This latter procedure reduced but did not completely eliminate the reactivity of bullfrog transferrin with antisaxiphilin antibodies (Fig. 5). The reduction in reactivity produced by further purification is consistent with the suggestion, stated above, that trace contamination by saxiphilin, on the order of ~0.1%, is the likely source of low level [^{3}H]STX binding observed for the transferrin preparation before the S-Sepharose step (Table 1). Because the two proteins were purified from the same source, trace contamination is an inherent problem that makes it difficult to establish whether the residual cross-reactivity observed in the experiment of Fig. 5 is a genuine reflection of antigenic similarity.

To pursue this relationship indirectly, several other transferrins from various species were also studied. We found that antisaxiphilin antibodies cross-reacted with different affinities with transferrins from species such as cow, human, and horse (Fig. 6A). Rabbit transferrin was essentially unreactive in this assay. Because these other transferrin samples contained no detectable [^{3}H]STX-binding activity and we have not observed saxiphilin-like activity in a mammalian species, it seems unlikely that this interspecies cross-reactivity is due to contamination by a saxiphilin-like protein. A similar pattern of species-specific cross-reactivity was observed when the test antigen concentration was increased at a fixed antibody concentration (Fig. 6B).

The antigenic relationship between saxiphilin and various

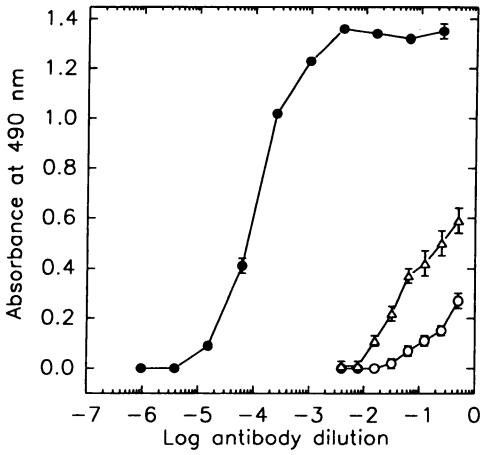

Fig. 5. Enzyme-linked immunoassay of saxiphilin and bullfrog transferrin with antisaxiphilin antibodies. Microtiter wells were coated with 250 ng of pure saxiphilin (●), bullfrog transferrin purified by chromatography on DEAE-Sephadex (△), or bullfrog transferrin subjected to an additional purification step of chromatography on S-Sepharose (○). The wells were then incubated with increasing dilutions of antisaxiphilin antibody and assayed using peroxidase-coupled second antibody, as described in Experimental Procedures. *Data points* for transferrin are the mean and standard deviation of six determinations. *Data points* for saxiphilin are the mean of duplicates.

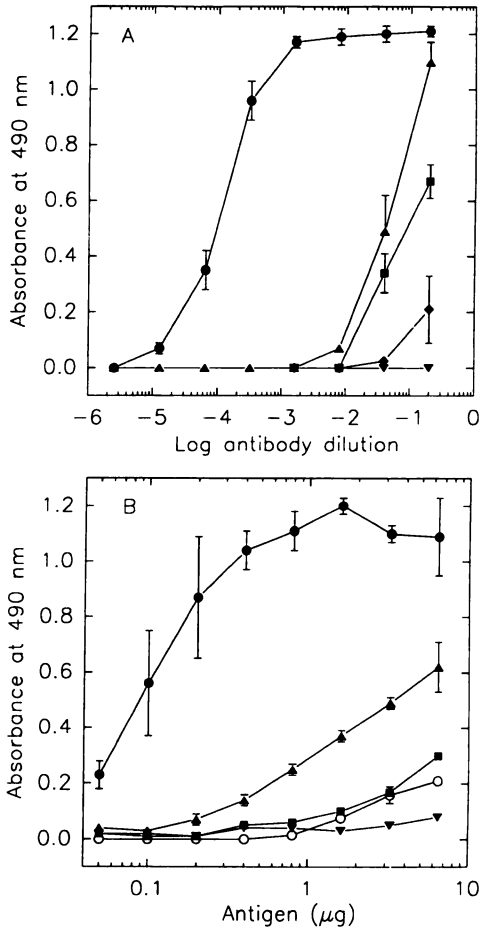

Fig. 6. Cross-reactivity of various transferrins with antisaxiphilin antibodies in an enzyme-linked immunosorbent assay. A, Microtiter wells coated with 250 ng of various antigens were incubated with increasing dilutions of antisaxiphilin antibody and assayed using peroxidase-coupled second antibody, as described in Experimental Procedures. B, Microtiter wells coated with increasing amounts of various antigens were incubated with a 1/2000 dilution of antisaxiphilin antibody and assayed using peroxidase-coupled second antibody. ●, Bullfrog saxiphilin; ▲, bovine transferrin; ■, human transferrin; ♦, horse transferrin; ▼, rabbit transferrin; ○, bullfrog transferrin after S-Sepharose chromatography. *Data points* are the mean and standard deviation of four determinations.

[1] L. Llewellyn and E. Moczydlowski, unpublished observations.

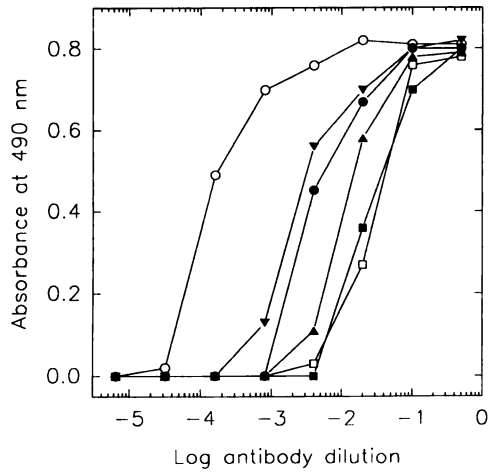

Fig. 7. Cross-reactivity of saxiphilin and various transferrins with anti-human transferrin antibodies in an enzyme-linked immunosorbent assay. Microtiter wells coated with 250 ng of various antigens were incubated with increasing dilutions of anti-human transferrin antibody and assayed using peroxidase-coupled second antibody as described in Experimental Procedures. ○, Human transferrin; ▼, rabbit transferrin; □, chicken ovotransferrin; ▲, bovine transferrin; ■, bullfrog transferrin; ●, bullfrog saxiphilin. *Data points* are the average of duplicate determinations.

transferrin proteins was also examined by testing, in a similar enzyme-linked immunosorbent assay, the reactivity of commercially obtained goat antibodies raised against human serum transferrin. In this experiment bullfrog saxiphilin was recognized with efficiency similar to that of various nonhuman transferrins, including those of rabbit, cow, and chicken (Fig. 7). Saxiphilin was actually somewhat more reactive than bullfrog transferrin in this assay. As judged by cross-recognition of frog saxiphilin and transferrins of several different species by polyclonal antibodies, saxiphilin behaves immunologically as a relative of the transferrin family.

Discussion

Results presented here, together with information obtained by partial sequencing of native saxiphilin (6) and recent cloning of saxiphilin cDNA,[2] establish that saxiphilin is a functionally and structurally unique member of the transferrin superfamily of proteins. High affinity binding of [^{3}H]STX is the property that originally led to the discovery of saxiphilin (4, 5, 23), but the functional significance of this interaction, if any, is presently unknown. Curiously, the phylogenetic distribution of saxiphilin appears to be limited to certain amphibians and reptiles that presently include *Rana catesbeiana* (bullfrog), *Rana sylvatica* (wood frog), *Bufo marinus* (marine or cane toad), *Ambystoma tigrinum* (tiger salamander), *Notophthalamus viridescens* (red spotted newt), *Taricha granulosa* (rough skinned newt), and *Thamnophis sirtalis* (garter snake).[3] Although the existence of STX and STX derivatives in the marine food chain is well documented (1, 24, 25), reports of STX in the terrestrial freshwater environment are limited to its production by a cyanobacterium, *Aphanizomenon flos-aquae* (26, 27). In contrast to the marine ecosystem, there is little information to suggest that STX plays a significant role in freshwater chemical ecology (28). Thus, the phenomenon of constitutive production by certain amphibians and reptiles of a plasma protein with high affinity for STX poses an interesting biological mystery.

Because partial sequencing of purified bullfrog saxiphilin previously revealed homology to the transferrin family of proteins (6), the purpose of the present work was to determine whether saxiphilin is derived directly from transferrin. Our results eliminate this possibility, because we have shown that saxiphilin is biochemically distinct from transferrin by a number of criteria. The saxiphilin protein is significantly larger than most transferrin proteins, migrating with an M_r of ~90,000 on SDS-PAGE, compared with ~78,000 for bullfrog transferrin (Fig. 3). Saxiphilin exhibits basic charge characteristics, as judged by an apparent pI of 10.7 (5), adsorption to an S-Sepharose cation exchange column (5), lack of adsorption to a DEAE-Sephadex anion exchange column (Fig. 1), and elution behavior on a chromatofocusing column (6). In contrast, bullfrog transferrin exhibits a pI in the range of 6.3–6.6 (12), which is consistent with adsorption to DEAE-Sephadex at pH 7.8 and low ionic strength (Fig. 1). Also, the two proteins clearly bind different ligands. Saxiphilin does not appear to bind $^{55}Fe^{3+}$ under conditions appropriate for transferrins, and purified samples of saxiphilin are colorless, implying the lack of spectroscopically active bound metal ions. Conversely, [^{3}H]STX does not bind to bullfrog transferrin or any of a large number of commercially available apotransferrins in our standard assay. The small amount of [^{3}H]STX binding detected for nominally pure transferrin from bullfrog (Table 1) can be attributed to trace contamination by saxiphilin (Fig. 5).

The conclusion that saxiphilin is not directly derived from bullfrog transferrin is also supported by the results of immunochemical experiments using polyclonal antibodies against saxiphilin and human transferrin (Figs. 5 and 6). Both of these antibodies clearly discriminate the two frog proteins. The demonstrated specificity of the antisaxiphilin antibodies suggests that they will be useful tools in immunohistochemical studies.

Apart from showing that saxiphilin and transferrin are different proteins, our immunochemical studies also confirm that they are structurally related. The extent of this structural relationship has recently been revealed by cloning of a cDNA from bullfrog liver that appears to correspond to an mRNA transcript of the coding sequence for saxiphilin.[2] This sequence information is helpful in interpreting the present immunological results. The saxiphilin cDNA clone predicts a secreted protein molecular weight of 90,818, which is consistent with the value reported in this paper (90,000 ± 3,000) for native saxiphilin determined by SDS-PAGE (Fig. 3). Except for one large gap due to a unique insertion of 144 residues in saxiphilin, pairwise sequence alignments of the deduced saxiphilin sequence with the sequences of various members of the transferrin family reveal amino acid sequence homology on the order of 51% identity with transferrin from *X. laevis* (African clawed frog) and 39–44% identity with various human transferrins (serum transferrin, lactoferrin, and melanotransferrin). Such sequence alignments show many short regions of almost complete identity and other regions of practically no homology between saxiphilin and various members of the transferrin family.[2] The immunological cross-reactivity observed here is consistent with such sequence homology and is typical of that found when polyclonal antibodies raised against one member of a protein family are assayed for reactivity with closely related

[2] M. Morabito and E. Moczydlowski. Cloning of bullfrog saxiphilin reveals a unique relative of the transferrin family that binds saxitoxin. Submitted for publication.

[3] L. Llewellyn, P. Bell, and E. Moczydlowski, unpublished observations.

members of the same protein family from other species. Such cross-reactivity may be expected to depend on the number of shared epitopes between related antigen proteins and the relative concentrations and affinities of various immunoglobulins in the polyclonal serum.

The cloned sequence of saxiphilin also explains the lack of Fe^{3+} binding reported in this paper. X-ray crystallography (10, 29) has shown that the two Fe^{3+}/HCO_3^- binding sites of transferrin are each formed by five highly conserved ligand residues, i.e., one aspartate, two tyrosines, one histidine, and one arginine. Alignment of the saxiphilin clone with the sequences of known transferrins reveals that only one of 10 of these critical residues is conserved in saxiphilin.[2] This finding leads to the prediction that both of the analogous binding domains of saxiphilin are nonfunctional with respect to Fe^{3+}, as confirmed here by the lack of detectable $^{55}Fe^{3+}$ binding.

In summary, saxiphilin may be recognized as a structural relative of the transferrin family that does not bind Fe^{3+}. Current information on this protein and its structural similarity to transferrin lead us to propose that saxiphilin may serve as a transport protein for an unidentified endogenous ligand or as an element of a detoxification system for a toxin acquired from the environment. The availability of antisaxiphilin antibodies described in this work and a cDNA clone encoding saxiphilin[2] will facilitate analysis of this protein and should help to elucidate its actual physiological role.

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