Cloning, Sequencing, and Recombinant Expression of the Porcine Inhibitor of Carbonic Anhydrase: A Novel Member of the Transferrin Family[†,‡]

Margot W. Wuebbens,[§] Eric D. Roush,[§,||] Carlos M. Decastro,[⊥] and Carol A. Fierke*[,§]

Department of Biochemistry, Box 3711, and Department of Medicine, Box 3250, Duke University Medical Center, Durham, North Carolina 27710

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ABSTRACT: The plasma from many vertebrates contains a component that specifically binds and inhibits carbonic anhydrase II with nanomolar affinity. Amino-terminal sequencing of pICA, the previously identified 79-kDa carbonic anhydrase inhibitor isolated from porcine plasma [Roush, E. D., & Fierke, C. A. (1992) *Biochemistry 31*, 12536−12542], and sequencing of four proteolytic fragments of pICA revealed that each of the partial sequences has 40−80% sequence identity with members of the transferrin protein family. We describe here the isolation of a full-length cDNA clone of pICA from a λgt11 porcine liver cDNA library. Heterologous expression of this cDNA clone in a *Pichia pastoris* expression system led to the secretion into the medium of 5 mg/L of a 79-kDa protein that specifically reacts with anti-pICA antibodies and binds tightly to a carbonic anhydrase−Sepharose affinity column. Pairwise sequential alignment of pICA with various transferrins reveals an amino acid identity as high as 64% and predicts that 16 transferrin disulfide bonds are conserved. However, despite these structural similarities, the properties of pICA are distinct from the properties of transferrin. pICA exhibits a significantly decreased affinity for iron that can be attributed to the loss of one of the eight amino acids that coordinate iron in the transferrins as well as both of the arginine residues responsible for anion binding. In addition, the antigenic determinants of pICA and the transferrins are not identical. These data imply that pICA, along with saxiphilin, is a member of a diverse superfamily of transferrin-like proteins with functions other than iron binding.

The carbonic anhydrases (CA)[1] are a family of zinc metalloenzymes that catalyzes the reversible hydration of carbon dioxide to produce bicarbonate and a proton (Christianson & Fierke, 1996; Dodgson, 1991; Silverman & Lindskog, 1988). This enzyme is ubiquitous in living systems, playing roles in CO_2 transport, secretory processes, calcification, and photosynthesis (Tashian, 1992). At least seven different mammalian isozymes of CA (CA I to CA VII) have been identified that vary in activity, tissue localization, and physiological role (Hewett-Emmett & Tashian, 1996; Tashian, 1992). Despite these differences, all vertebrate CAs sequenced to date share at least 30% homology at the amino acid level. Two isozymes, CA I and CA II, are present in high concentrations in red blood cells and in lower concentrations in the cytosol of many tissues (Dodgson, 1991). A CA II deficiency syndrome exists in humans, leading to osteopetrosis, renal tubular acidosis, mental retardation, growth retardation, and cerebral calcification (Sly & Hu, 1995).

CA inhibitory activity has been observed in the sera of a number of mammals including pigs, rats, cats (Booth, 1938), sheep (Leiner et al., 1962), dogs (Rispens et al., 1985), and rabbits (Hill, 1986). The protein responsible for this inhibitory activity in porcine plasma, porcine inhibitor of carbonic anhydrase (pICA), has been previously purified to electrophoretic homogeneity using a combination of anion-exchange and CA II affinity chromatography (Roush & Fierke, 1992). pICA is a monomeric, N-glycosylated, 79-kDa protein that reversibly inhibits the CA II isozyme with a 1:1 stoichiometry. This protein is a very efficient and specific inhibitor of porcine CA II, with a $K_i \approx 0.2$ nM at physiological pH (Roush & Fierke, 1992). The concentration of pICA in porcine plasma ($\approx 1\ \mu$M) is significantly higher than the K_i, indicating that any porcine CA II leaked into the plasma is complexed with pICA. Similarly, in rat plasma a complex between CA II and a protein with a molecular mass comparable to pICA has been observed (Ojteg & Wistrand, 1994). Formation of this complex prevents plasma CA II from being filtered by the kidneys into the urine (Appelgren et al., 1989; Ojteg & Wistrand, 1994).

To further illuminate the biochemical properties and physiological function of this inhibitor, we have isolated and

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* Author to whom correspondence should be addressed. Phone (919) 684-2557; Fax (919) 684-8885; E-mail fierke@biochem.duke.edu.

[§] Department of Biochemistry.

[||] Current address: Department of Anesthesiology, Box 3094, Duke University Medical Center, Durham, NC 27710.

[⊥] Department of Medicine.

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[1] Abbreviations: BSA, bovine serum albumin; CA I, II, etc., carbonic anhydrase isozyme I, II, etc.; hCA II, human carbonic anhydrase isozyme II; buffer A, 25 mM Tris-SO_4, pH 8; Fe^{3+}NTA, ferric dinitrilotriacetic acid; ICP, inductively coupled plasma emission spectroscopy; Lf, lactoferrin; hLf, human lactoferrin; pLf, porcine lactoferrin; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline plus 0.05% Tween-20; PCR, polymerase chain reaction; pICA, porcine inhibitor of carbonic anhydrase; sax, saxiphilin; SDS, sodium dodecyl sulfate; Tf, transferrin; bTf, bovine transferrin; hTf, human transferrin; pTf, porcine transferrin.

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sequenced a full-length cDNA clone of pICA from a porcine liver library. Furthermore, we have recombinantly expressed this cDNA clone in the yeast *Pichia pastoris*. At both the amino acid and nucleotide levels, the pICA clone exhibits a high degree of sequence identity with members of the transferrin or siderophilin family of iron binding proteins [for review, see Aisen (1994), Crichton and Ward (1992), and Welch (1992)]. Each of these monomeric glycoproteins of ≈80 kDa has the capacity to tightly bind two Fe^{3+} molecules concomitantly with two HCO_3^{2-} ions (Aisen, 1994). Crystallographic and amino acid sequence analyses have shown that the ≈680-residue chain of each of these proteins can be divided into two homologous halves with 35−40% identity (Mazurier et al., 1983; Metz-Boutique et al., 1984) and that each half binds a single anion and iron atom (Anderson et al., 1989, 1990; Bailey et al., 1988). The three major categories of transferrins include serum transferrin (Tf), present in plasma and extracellular fluids; lactoferrin (Lf), detected in milk, a variety of secreted fluids, and the granules of neutrophil granulocytes; and ovotransferrin, found in egg whites.

At the amino acid level, the sequence for pICA reported here is 63% identical to porcine Tf (pTf) (Baldwin & Weinstock, 1988) and 60% identical to porcine Lf (pLf) (Alexander et al., 1992). However, despite this high degree of similarity, pICA is biochemically and functionally distinct from the transferrins in a number of ways. Specifically, while pICA inhibits CA II with a nanomolar K_i (Roush & Fierke, 1992), all transferrins tested fail to inhibit or bind to CA II at micromolar concentrations. In addition, pICA does not bind iron with high affinity, and the major determinants of antibody binding to pICA are antigenically distinct from those of pTf and other transferrins. In these latter two respects, pICA is similar to the protein saxiphilin. This recent addition to the transferrin family is found in bullfrog plasma and binds the neurostimulatory toxin saxitoxin (Li et al., 1993). Although the full-length cDNA clone shows that saxiphilin has >50% primary sequence identity with some transferrins (Morabito & Moczydlowski, 1994), saxiphilin does not bind Fe^{3+}, and it shares a limited number of antigenic determinants with the transferrins (Li et al., 1993). Thus, pICA and saxiphilin are members of a growing family of proteins with transferrin-like sequences whose function is to transport or sequester molecules other than Fe^{3+}.

MATERIALS AND METHODS

Materials. pICA was purified by anion-exchange and affinity chromatography as described previously (Roush & Fierke, 1992). Recombinant human CA II (hCA II) from *Escherichia coli* (Nair et al., 1991) was purified using sulfonamide affinity chromatography (Osborne & Tashian, 1975), and the concentrations of these proteins were determined as previously described (Roush & Fierke, 1992). Apo and holo forms of bovine Tf (bTf), human Tf (hTf), and human Lf (hLf) were obtained from Sigma. pICA−Sepharose and pTf−Sepharose were prepared from CNBr-activated Sepharose 4B (Sigma) using methods previously described for the preparation of hCA II−Sepharose (Roush & Fierke, 1992). Equilibrium dialysis chambers and membranes were from Bel Art. Centriprep, Centricon, and Microcon concentrators were from Amicon.

Molecular weight markers, prepoured SDS−polyacrylamide gel electrophoresis (SDS−PAGE) gels, nitrocellulose paper, as well as mini protein electrophoresis, mini trans-blot, and slot blot equipment were purchased from Bio-Rad. Sequagel concentrate was from National Diagnostics. Rabbit anti-pICA serum was produced by Pocono Rabbit Farms, Canadensis, PA. The λgt11 porcine liver cDNA library was purchased from Clontech. DNA primers for sequencing and polymerase chain reaction (PCR) amplification were prepared by the Botany Department, Duke University, or by Bio-Synthesis, Inc. Sequenase T7 DNA polymerase and radionucleotides were purchased from Amersham/U.S. Biochemical Corp. Polynucleotide kinase and chromatography resins were supplied by Pharmacia Biotech. Materials for TA cloning and expression of recombinant protein in *Pichia pastoris* were obtained from Invitrogen. *Taq* polymerase, T4 DNA ligase, and *Eco*RI were supplied by Life Technologies. pBluescript II KS^+ and *E. coli* XL1-Blue cells were purchased from Stratagene. Reinforced nitrocellulose was obtained from Amersham or Schleicher & Schuell. All other chemicals were reagent-grade from standard suppliers. Double-stranded DNA sequencing was performed according to Sanger et al. (1977) using Sequenase 2.0. pTf was purified from porcine plasma using a four-step purification derived from the work of Chung et al. (1991).

Protein Sequencing of pICA. pICA was digested with endoproteinase Lys-C, the proteolytic fragments were purified by reverse-phase high-pressure liquid chromatography, and selected fragment peaks, as well as the amino terminus of the intact protein, were sequenced at the Harvard Microchemistry Facility (Cambridge, MA) using automated Edman degradation. Peptide sequences generated by this method were then screened against the Protein Data Bank, Protein Identification Resource, SwissProt, and GenBank libraries of known protein sequences using BLAST (Altschul et al., 1990).

PCR Isolation of a Partial pICA Clone. The following nondegenerate oligonucleotides were designed from the pICA peptide sequences using mammalian codon preferences sense Lys1 (aag ccc gtg gtg gcc gag ttc tac gg) and antisense Lys2 (ac gaa ggc cac gtc gcc cac gtc ctc ctg). These primers were used to amplify a porcine liver λgt11 cDNA library using low stringency conditions: 35 cycles, 8 ng/μL template DNA, 1.5 mM $MgCl_2$, 36 °C annealing temperature, polymerization = 30 s, annealing and melting cycles = 1 min. The reaction products were separated by electrophoresis on a 1.5% low-melting agarose gel, and a slice corresponding to dsDNA of 300−500 bp in length was excised and reamplified under more stringent conditions using the same primers (30 cycles, 1 mM $MgCl_2$, 45 °C annealing temperature, polymerization = 25 s). The products of the second PCR amplification were ligated into the *Eco*RI site of the pCR II vector (Invitrogen). Isolation and dideoxy sequencing (Sambrook et al., 1989) of a number of clones yielded a 0.4-kb putative subclone of pICA designated EDR14.

Isolation of Full-Length cDNA for pICA. E. coli Y1090 cells were infected with the same λgt11 library used for PCR amplification, and a total of 3×10^5 plaques were lifted onto duplicate nitrocellulose filters by standard procedures (Sambrook et al., 1989). The filters were baked at 80 °C for 2 h and then prehybridized for 5 h at 65 °C in 6× SSC, 2× Denhardt's solution, 0.1% SDS, 1× SSPE, and 100 μg/mL sheared salmon sperm DNA (Sambrook et al., 1989). The oligonucleotide probe-14 (gatacttcttcctcctgactcaggggaag) was labeled with [γ-^{32}P]ATP using polynucleotide kinase under

standard conditions (Sambrook et al., 1989) and then added to the prehybridization solution for overnight incubation with the filters at 65 °C. After being washed once in 2× SSC and 0.05% SDS at 42 °C for 2 h, twice at 65° for 20 min, and then once in 1× SSC and 0.05% SDS at 65 °C for 20 min, the filters were air-dried and exposed to Kodak XAR-5 film overnight. Agar plugs corresponding to the positions of likely positive plaques were removed from the original plates, and the phage in these plugs were replated and screened a second time under similar conditions to yield a total of 11 pure, positive plaques. The phage isolated from these plaques were regrown, and Promega λ-sorb was used to purify the phage DNA. This was digested with *Eco*RI and the insert DNA fragments were gel-purified and subcloned into the *Eco*RI site of pBluescript II. The resulting plasmids were transformed into *E. coli* XL1-Blue cells and purified, and the inserts were sequenced (Sambrook et al., 1989). Two subclones, MW6L and MW9b, were identified that together encompass the entire open reading frame of pICA and define an internal *Eco*RI site with their 3′ and 5′ ends, respectively. The two plasmids containing these inserts were digested with *Eco*RI, and the inserts were ligated in the correct orientation to yield pMW15, which contains the entire pICA open reading frame in the pBluescript vector.

Site-Directed Mutagenesis and Expression of Full-Length pICA in P. pastoris. To subclone the cDNA encoding mature pICA into the yeast secretion vector, the internal *Eco*RI site was deleted and a new *Eco*RI site was simultaneously added at the beginning of the mature protein using oligonucleotide-directed mutagenesis (Kunkel, 1985; Kunkel et al., 1987). For this, pMW15 was transformed into the CJ236 strain of *E. coli* (Bio-Rad) followed by infection with the helper phage M13K07 to produce single-stranded DNA. Oligonucleotide DelEcoR949 (gtcgtggcagagttctatgggtca) was used to delete the internal *Eco*RI site at position 949, while oligonucleotide AddEcoR700 (ctggggctgtgtctggaattccctaaggaaactgtg) was used to add an *Eco*RI site at position 700. Neither of these substitutions altered the amino acid sequence of mature pICA. The insert from the resulting plasmid, pMW16, was then excised with *Eco*RI and ligated into the corresponding site of the *P. pastoris* secreted expression vector pPIC-9 to yield pPicMW16.

Preparation of spheroplasts of the *P. pastoris* strain GS115 using zymolase and subsequent transformation with pPicMW16 were carried out as described by Invitrogen. Transformants were patched onto both minimal dextrose and minimal methanol plates, and those colonies exhibiting poor growth on methanol were tested for expression of pICA. For this test, cells were cultured in 250 mL of growth medium containing 20 g of peptone, 10 g of yeast extract, 12 g of yeast nitrogen base without amino acids, 0.1 M potassium phosphate buffer, pH 6.0, 0.4 mg of biotin, and 10 mL of glycerol per liter with vigorous shaking in 2-L baffled flasks at 30 °C. The cells were harvested at OD_{600} of 2−6 by centrifugation (1500*g*, 10 min) and were resuspended in 50 mL of the expression medium described above where 5 mL/L of methanol was substituted for the glycerol. Incubation was continued for 4 days with vigorous shaking in a baffled 250-mL flask at 30 °C with the addition of 250 μL of methanol each day after the first. Cells were pelleted, and the media were filtered through Costar 0.2-μm filters prior to storage at 4 °C. To test for pICA expression, the media were concentrated 10-fold in a Microcon 30 apparati with concomitant exchange of buffer to 25 mM Tris-SO_4, pH 8.0 (buffer A). Aliquots of the concentrated samples were then electrophoresed on a denaturing 12% polyacrylamide gel before transfer to nitrocellulose for probing with anti-pICA specific antibodies.

Purification of Recombinant pICA. A 6-mL aliquot of expression medium was diluted 33-fold into 1 mM EDTA and applied to a DEAE Affi-Gel Blue column (2 mL) equilibrated with buffer A. After the column was washed with buffer A, the sample was eluted with a linear gradient of 0−100 mM $(NH_4)_2SO_4$ in buffer A. Those fractions containing recombinant pICA, as assayed by SDS−PAGE analysis, were pooled and concentrated to 1.2 mL in a Centricon 30. Alternatively, a 5-mL aliquot of medium was diluted with 10 mL of buffer A and passed twice through a 1-mL hCA II−Sepharose column equilibrated with buffer A. Proteins were eluted with 8 mL of 1.0 M NaSCN in buffer A, and the eluant was dripped directly into 25 mL of stirring buffer A. This sample was then exchanged into buffer A and concentrated ≈830-fold by dialysis and Centricon concentration.

Affinity Purification of Anti-pICA Antibodies. To obtain anti-pICA-specific antibodies, a 3.5-mL aliquot of anti-pICA serum was diluted with phosphate-buffered saline (PBS) to a final volume of 10 mL and then loaded onto a 3-mL column of pICA−Sepharose equilibrated with PBS at 4 °C. The column was washed with 15 mL of cold PBS, pICA-specific antibodies were eluted with 100 mM glycine, pH 2.8, and a total volume of approximately 4 mL was pooled by absorbance at 280 nm. After neutralization with 1 M Tris, pH 8.0, the antibodies were frozen at −20 °C. To remove antibodies that cross react with pTf, a 0.3-mL aliquot of the affinity-purified antibody was agitated gently overnight at 4 °C with 0.5 mL of pTf−Sepharose. The resin was then pelleted, and the supernatant was removed.

Immunochemistry. For Western blots, protein samples were transferred from SDS−PAGE gels to nitrocellulose paper following the manufacturer's instructions for a 1 h transfer at 100 V (Bio-Rad). Slot and Western blots were first blocked for nonspecific antibody binding by incubation for 1 h in PBST/1% Blotto and then washed in PBST followed by incubation with a 1:50 dilution in PBST of a 0.3 mL aliquot of the primary antibody. After again being washed with PBST, the filters were incubated for 1.5 h with a 1:300 dilution of a donkey, anti-rabbit, horseradish peroxidase-linked, whole antibody reagent (Amersham). After a final wash with PBS, the blots were developed by incubation with 15 mL of 0.5 mg/mL 4-chloro-1-naphthol, 83% PBS, and 17% methanol containing 7 μL of 30% hydrogen peroxide. Each wash was 3-fold and 5 min in duration, and washes as well as incubations were performed at room temperature on a rocking platform.

Inhibition of CA II Activity. The CO_2 hydrase activity of hCA II was measured using a pH indicator assay (Khalifah, 1971) in a stopped-flow spectrophotometer from KinTek Instruments with the following buffer conditions: 14 mM 1,2-dimethylimidazole, pH 8.0, 24 μM *m*-cresol purple, and 1 mM EDTA, 25 °C. Inhibition of hCA II activity was measured by preincubating the inhibitor sample and hCA II in the colorimetric buffer at 25 °C for at least 1 min, diluting 2.5-fold into CO_2-saturated water, and then assaying CO_2 hydrase activity (Roush & Fierke, 1992).

Table 1: Amino Acid Sequences of pICA Peptide Fragments

peptide	sequence	% identity to pTF
N-Term	FPKET VRW?T VSSQE	50
Lys1	RTSYL ECIRA ILANE ADAVT IDGGL VFEAG LAPYN LKPVV AEFYG	80
Lys2	CLQED VGDVA FVRHV TVFEN LPDKA DRDQY ELLCK	81
Lys3	LYLGY EYFAA IQHLR RVQG? EEPQR VMWCA VGQHE RTK	40
Lys4	DLKQE DFQLL CPDGT RKPVT EAQSC HLAAV PSHAV VSRKD	63

Inductively Coupled Plasma Emission Spectroscopy. A sample of 40 μM pICA purified from porcine plasma was dialyzed overnight against water. Aliquots of 0.9 mL of both the protein and the dialysate were analyzed by 31-element ICP at the Chemical Analysis Laboratory, Institute of Ecology, University of Georgia, for the following elements: Ag, Al, As, B, Ba, Be, Bi, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Pb, Sb, Se, Si, Sn, Sr, U, V, W, Y, and Zn.

Equilibrium Dialysis and Colorimetric Assay for Fe^{3+} Binding. A 1 mM stock solution of Fe^{3+}NTA (1:4) was prepared using a 2.7% ferric chloride standard from Sigma (Zapolski & Princiotto, 1980). Samples of 10 μM pICA, apo-hTf, or holo-hTf in 1 mL of 50 mM Tris-Cl, pH 8.0, 10 mM $NaHCO_3$ were equilibrated for 40 h at room temperature against an equal volume of the same buffer in an equilibrium dialysis chamber, or against the same buffer with the addition of 100 μM Fe^{3+}NTA. Samples from each chamber were then assayed for iron using the ferrozine-based colorimetric assay of Fish (1988). Extinction coefficients at 280 nm of 87 000 and 114 000 M^{-1} cm^{-1} were used to determine the concentrations of apo- and holo-hTf, respectively (Ross et al., 1995).

RESULTS AND DISCUSSION

Primary Amino Acid Sequencing of pICA. To further investigate the biochemical and structural properties of pICA, the amino acid sequences of the N-terminus of intact pICA and four fragments generated by digestion with endoproteinase Lys-C were determined. These results are shown in Table 1. Comparison of these sequences with protein sequences in a number of protein databases (Protein Data Bank, PIR, SwissProt, and GenBank) revealed a high degree of sequence identity to members of the transferrin family from a variety of species. The highest amount of sequence identity was observed between the pICA peptides and similar fragments from pTf (see Table 1). Any non-transferrin protein identified by the algorithm used to search the various sequence databases (BLAST; Altschul et al., 1990) as sharing sequence identity with the peptide sequences of pICA had the following properties: the matching amino acid word scores were low (<55), the probability of random matching was high ($>20\%$), and the protein was similar to only one pICA peptide.

Cloning and Sequencing of pICA. The initial step in the cloning of pICA was PCR amplification of a commercial λgt11 porcine liver cDNA library using primers derived from the amino acid sequences of pICA peptide fragments Lys1 and Lys2 (Table 1). This procedure yielded a 0.4-kb putative pICA subclone (EDR14) that demonstrated a high level of sequence identity to a portion of the coding regions of both pTf and pLf. The nucleotide sequence of EDR14 was then used to further screen the cDNA library for a full-length pICA cDNA clone. Initial screening with probes obtained by randomly priming EDR14 yielded only a large number of pTf and pLf clones, consistent with the overall $>70\%$ sequence identity between the cloned sequence and the reported cDNA sequences for these two proteins (Alexander et al., 1992; Baldwin & Weinstock, 1988).

In order to screen the library more selectively, we designed an oligonucleotide probe corresponding to the region of EDR14 with the least sequence identity to pTf and pLf. Filter screening of the λgt11 library with this probe yielded 11 positive plaques. In three instances, two inserts were observed after *Eco*RI digestion of the purified phage DNA, suggesting the presence of an internal *Eco*RI site within the pICA cDNA. Subsequent nucleotide sequencing of a number of the inserts verified this supposition and yielded the entire open reading frame of pICA. Two inserts, MW6L and MW9b, were joined at their 3′ and 5′ end *Eco*RI sites, respectively, in the pBluescript vector to create pMW15. Both strands of the insert in this plasmid were sequenced and the nucleotide sequence as well as the deduced amino acid sequence for pICA are shown in Figure 1. (This nucleotide sequence has been entered into GenBank and been assigned accession number U36916.) Comparison of the N-terminal amino acid sequence of native pICA (Table 1) with the sequence deduced from the pICA cDNA indicates the presence of a 19-residue signal sequence. The 2945-bp cDNA clone contains 646 bases of 5′ untranslated sequence followed by the pICA open reading frame of 704 amino acids that begins with the ATG for Met-19 at nucleotide 647 and ends with a TAA termination codon following Asn685. The 181 bases 3′ to the open reading frame contain a consensus polyadenylation sequence (AATAAA) that is 23 bases upstream of the poly(A) tail.

The 645 nucleotides of MW15 located 5′ of the pICA start codon contain six ORFs which vary in length from 71 to 141 amino acids. Two of these contain a Met near the 5′ end of the ORF and could, therefore, be actively transcribed regions. ORF1, a 75 amino acid reading frame, extends from nucleotide 462 to 238 on the complementary strand. A search of the protein databases (Altschul et al., 1990) revealed very little sequence homology between ORF1 and any previously reported ORFs or protein sequences (high scores ≤ 60). ORF2 is also located on the complementary strand and extends from nucleotide 601 to 224 (126 amino acids). This sequence shows a high degree of similarity (high scores range from 234 to 70) to a number of chemokines, including macrophage inflammatory proteins 1α and 1β (Widmer et al., 1993), and may be a porcine equivalent of one of these proteins.

The identity of the cloned sequence as that of pICA is supported by a number of points. The existence of a single consensus sequence for glycosylation (NXS/T) at positions 472−474 in the cloned pICA amino acid sequence is

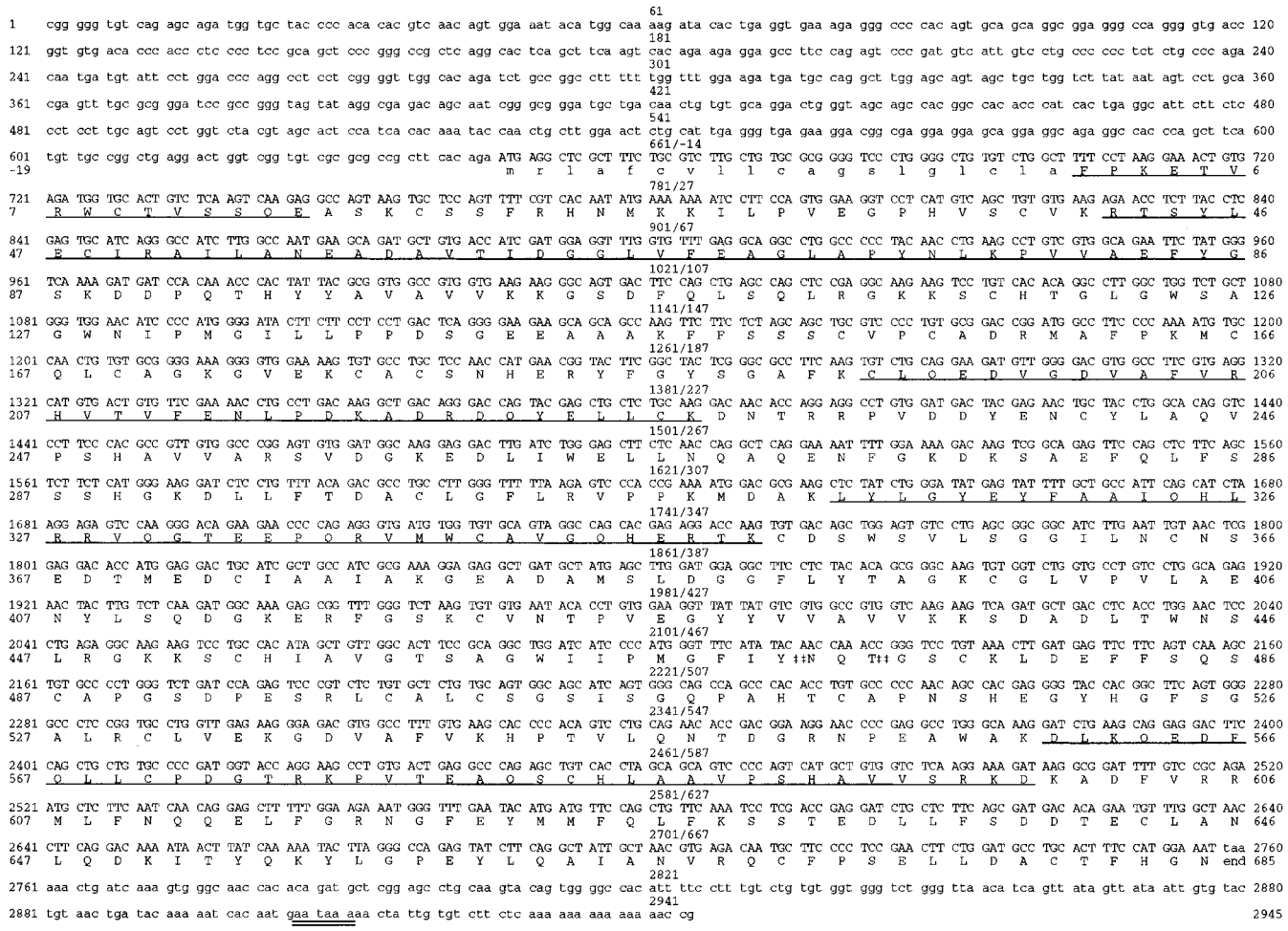

FIGURE 1: Nucleotide and deduced amino acid sequences of the MW15 cDNA clone of pICA. The 19 amino acid sequence corresponding to the leader sequence appears in small letters preceding the mature NH_2-terminal phenylalanine at position 1. Solid underlines correspond to the amino acid sequences of the pICA peptides listed in Table 1. The double underline indicates the position of the polyadenylation sequence, and the consensus site for N-linked glycosylation at amino acids 472−474 is enclosed in ‡‡. The internal *Eco*R I site is located between nucleotides 949 and 954.

consistent with the observation that native pICA contains approximately 4 kDa of N-linked glycosylation (Roush & Fierke, 1992). In addition, the predicted molecular weight of the nonglycosylated, 685-residue mature protein encoded by the MW15 nucleotide sequence is 75 674, which corresponds well to the value of 75 000 that has been estimated for the native, nonglycosylated protein by SDS−PAGE and mass spectrometry (Roush & Fierke, 1992). The strongest evidence for the correlation between pICA and the cloned sequence is the presence of both the pICA amino-terminal sequence and all four fragments generated by proteolytic cleavage of pICA within the predicted amino acid sequence of MW15. These five sequences are widely scattered throughout the cloned sequence, adding further weight to the identity of the clone. One position of natural heterogeneity was observed during nucleotide sequencing of partial pICA clones obtained from the same library as the full-length clone. The substitution of a C for the G at position 1834 in two of these clones resulted in a predicted amino acid change of Ala → Pro at position 378.

Expression of Recombinant pICA. Previous attempts at recombinant expression of members of the transferrin family in prokaryotic expression systems have proven unsuccessful (Aldred et al., 1984; Funk et al., 1990; Steinlein et al., 1995), possibly due to the large number of disulfide bonds that must be correctly formed for proper folding of these proteins. However, recombinant transferrin has been expressed in eukaryotic systems using the pNUT vector in cultured baby hamster kidney cells [10−30 mg/L of the amino-terminal half-molecule of hTf (Funk et al., 1990; Woodworth et al., 1991) and 125 mg/L of full-length hTf (Mason et al., 1993)]. In addition, the N-terminal half of hTf has also been expressed in *P. pastoris* at levels exceeding 50 mg/L (Steinlein et al., 1995). Due to the similarity of the pICA cDNA sequence to members of the transferrin family, we chose to use the eukaryotic *P. pastoris* system for recombinant expression of pICA. This yeast system combines the advantages of a eukaryotic system with regard to protein folding and posttranslational modification with the relative ease of growth and genetic manipulation of prokaryotic systems as well as avoiding the problem of contamination of the expressed protein with bovine Tf and Lf from fetal calf serum in tissue culture medium (Funk et al., 1990). In *P. pastoris* cells grown on methanol, approximately 5% of the poly $(A)^+$ RNA is derived from the alcohol oxidase (AOX1) gene (Ellis et al., 1985). To obtain heterologous expression in this system, the gene of interest is cloned behind the AOX1 promoter in a plasmid vector and then integrated into the yeast genome by homologous recombination (Cregg et al., 1993).

Since native pICA is a secreted protein, the *P. pastoris* expression vector pPic-9 was chosen for expression of

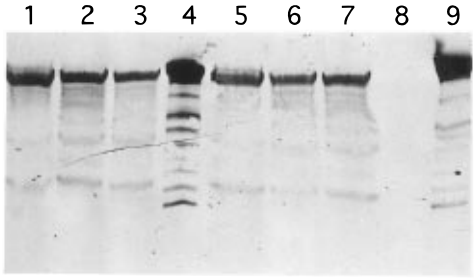

FIGURE 2: Western blot of *P. pastoris* expression media. Lanes 1−3 and 5−7 contain the concentrated expression medium from six different pPicMW16 transformants. Lane 8 contains the expression medium from a *P. pastoris* control strain that expresses BSA at ≈ 1 g/L. Lanes 4 and 9 contain 0.2 μg of pICA standard.

recombinant pICA. In this plasmid, the multiple cloning site follows the secretion signal sequence from the *Saccharomyces cerevisiae* α-factor prepropeptide, which allows for secretion of the protein of interest into the medium (Cregg et al., 1993). Prior to subcloning the pICA coding region into this vector, the internal *Eco*RI site of the MW15 insert was deleted and a new *Eco*RI site situated directly at the two amino acids bounding the cleavage site for the native pICA signal sequence was inserted using oligonucleotide-directed mutagenesis (Kunkel, 1985; Kunkel et al., 1987). The resulting insert, MW16, was cloned into the *Eco*RI site of pPic-9 to yield pPicMW16. Using this construct, secreted expression of pICA followed by cleavage of the α-factor secretion signal will produce mature, wild-type pICA containing an additional 7 amino acids (EAEAYVE) at the amino terminus.

After transformation of pPicMW16 into the GS115 strain of *P. pastoris*, we isolated a number of Mut^s (methanol utilization slow) colonies. The conversion from Mut$^+$ to Mut^s indicates the integration of linearized pPicMW16 into the yeast chromosome at the AOX1 gene. The methanol induction media from the growth of approximately 30 such Mut^s transformants were tested for the presence of pICA by Western blotting. Typical results are shown in Figure 2. All of the media tested exhibited low levels of expression of a protein with a molecular weight identical to that of native pICA that was recognized by affinity-purified, anti-pICA antibodies. The complete lack of any cross-reacting material in the medium from a control *P. pastoris* strain engineered to express bovine serum albumin (BSA) upon methanol induction (lane 8) further verifies the identity of this protein as recombinant pICA rather than a native cross-reacting *P. pastoris* protein. By comparison with known amounts of native pICA, the amount of recombinant pICA expression was estimated to be ≈5 mg/L of medium. The relatively low level of pICA expression was not due to incomplete transport of expressed protein into the media since no pICA was detected in a Western blot of whole cell extracts (data not shown). The level of recombinant pICA expression was not increased by altering the induction time or medium formulation. The secreted, recombinant pICA bound to both resins employed for the purification of the native protein from porcine plasma (Roush & Fierke, 1992). As can be seen from Figure 3, while chromatography on DEAE Affi-Gel Blue did not result in an appreciable purification of the expressed protein, binding to and elution from hCA II−Sepharose produced complete separation of recombinant pICA from all other detectable medium proteins.

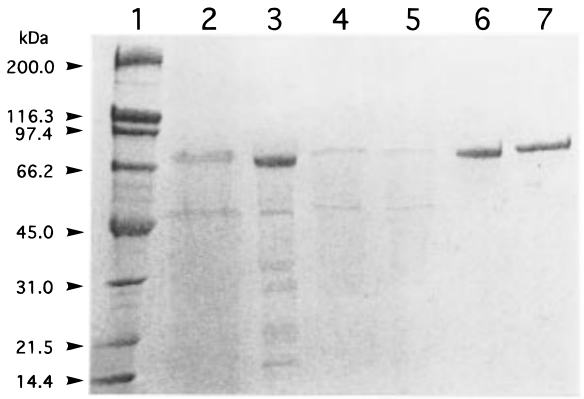

FIGURE 3: SDS−PAGE of the purification of expressed pICA. Lane 1: Molecular weight standards. Lane 2: Undiluted expression medium. Lane 3: Concentrated DEAE Affi-Gel Blue eluant. Lane 4: Diluted medium applied to the hCA II−Sepharose column. Lane 5: hCA II−Sepharose flowthrough. Lane 6: Concentrated hCA II−Sepharose eluant. Lane 7: 1.2 μg of pICA standard. A sample volume of 30 μL was used for all lanes, and proteins were stained with Coomassie blue dye.

Sequence Comparison of pICA and the Transferrin Family. Pairwise alignment of the predicted amino acid sequence of pICA with that of hTf (Yang et al., 1984), pTf (Baldwin & Weinstock, 1988), pLf (Alexander et al., 1992), and saxiphilin (Morabito & Moczydlowski, 1994) indicates 64%, 63%, 60%, and 41% identity, respectively, with these proteins. The multiple sequence alignment of all five of these proteins is shown in Figure 4. (For this alignment, the 144 amino acid insert in saxiphilin was not included.) As can be seen from this figure, the similarities between pICA and the transferrins extend throughout the pICA amino acid sequence and definitively identify pICA as the newest member of this family. The previously identified members serve a variety of functions. Tf is a vital component in the intercellular transport of iron while Lf and ovotransferrin are believed to act as bacteriostatic agents in milk and egg white, respectively, by preventing bacteria from accessing the Fe^{3+} required for their growth (Crichton & Ward, 1992). Lf has also been implicated in a variety of functions unrelated to iron binding [for review, see Iyer and Lonnerdal (1993)]. A fourth class of transferrins, the melanotransferrins, has been identified in the membranes of malignant melanoma cells. Melanotransferrin exhibits ≈40% homology with other transferrins (Rose et al., 1986), is antigenically similar to Tf and Lf (Brown et al., 1982) but has only a single functional iron binding site located in its N-terminal domain (Baker et al., 1992). Saxiphilin, the fifth member of the family, is a 91-kDa protein originally purified from bullfrog plasma as a result of its high affinity for the neurotoxin saxitoxin. Partial amino acid sequencing and subsequent cloning of the cDNA for saxitoxin verified that it is a member of the transferrin family, despite the fact that saxiphilin does not bind iron (Li et al., 1993; Morabito & Moczydlowski, 1994).

As stated previously, comparison of the N-terminal amino acid sequence of native pICA with that deduced from the pICA cDNA indicates the presence of a 19-residue signal sequence. Similar secretory signal sequences have been identified in all known transferrin cDNA sequences (Crichton & Ward, 1992; de Jong et al., 1990). The presence of such a leader sequence is consistent with the localization of pICA and the transferrins in serum or secreted fluids. The existence of a single site for N-linked glycosylation within

```
     -19                1        a          b                   b         a              Δ
hTf  MRLAVGALLVCAVLG LCLAVP-DKTVRWCA VSEHEATKCQSFRDH MKSVIPSDGPSVACV KKASYLDCIRAIAAN EADAVTLDAGLVYDA  70
pTf  --------------- ----V.-QKTVRWC. iS..EA.KC.SFRen M...v.n-GP.VsCV KKsSYLDCI.AI... EADAVTLDAGLVfeA  69
pLf  MkL.i.ALL..g.LG LCLA.P-KK.VRWC. iS..E.sKCr.Wq.k .r...P.-----.Ci rrAS..DCIRAIAA. .ADAVTLDgGLVfeA  65
sax  M.......L...ii. L..A.P.AK.VRWCA iSd.E..KC..... ......t----l.CV .rsS..DC.TAI... qADAm.LDsG.VYeA  67
pICA MRLA...LL..g.LG LCLA.P-KeTVRWC. VS..EAsKC.SFR.n MK.ilP.eGP.VsCV Kr.SYLeCIRAI.AN EADAVTiDgGLVfeA  70

                                  Δ                        c     ↓
hTf  YLAP.NLKPVVAEFY GSKEDPQ-----TFY YAVAVVKKDSGFQMN QLRGKKSCHTGLGRS AGWNIPIGLLYCD-- LPEPRKP---LEKAV  150
pTf  .LAPYNLKPVVAEFY G.KdnPQ-----T.Y YAVAVVKK.S.FQ.N QLqGKrSCHTGLGRS AGW.IP.GLLY.-- LPEPRKP---iEKAV  149
pLf  --..YKLrPV.AE.Y GteEnPQ-----TyY YAVAVVKK...FQ.- QLqGrKSCHTGLGRS AGWNIPIGLL...-- L.....Pe.-LqKAV  144
sax  ...PYNLKPiiAE.Y .S..D.Q*****T.h YAVAmVKK.SaFQ.N QLkGKrSCHsGv.kt .GW...v.vL..... L......k..iqrAM  296
pICA .LAPYNLKPVVAEFY GSKdDPQ-----T.Y YAVAVVKK.S.FQls QLkGKKSCHTGLG.S AGWNIP.GIL..--- -Pd..------EeA.  145

          d  e          g  d         g e          Δ      c                                    f
hTf  ANFFSGSC.PCADGT DFPQLCQLCPG---- -CGCSTL.QYFGYSG AFKCLKDGAGDVAFV KHSTIFENLANKADR DQYELLCLDNTRKPV  235
pTf  AsFFS.SCvPCAD.. nFPkLCQ.C.GkGAE KCACS..E.YFGYaG AF.CLKe.AGDVAFV KHSTv.ENL.dKADR DQYELLC.DNTRrPV  239
pLf  A.FFS.SCvPCADG. .yP.LCQLC.GkG.d KCACSs.E.YFGYSG AF.CLh.G.GDVAFV K.STvFENL..KADR DkYELLC.DNTRKPV  234
sax  s.FFS.SCiP.A..T n---LCk.C.Ge.gk .C..S..E.YyG..G AFrCLKe..GDVAFl r.t.l.d------... e.YELLC.DNTRKPl  378
pICA A.FFS.SCvPCAD.. .FPkmCQLC.GkG.E KCACS..ERYFGYSG AFKCLqe..GDVAFV rH.TvFENL.dKADR DQYELLC.DNTRrPV  235

         f       Δ
hTf  DEYKDCHLAQVPSHT VVARSMGGKEDLIWE LLNQAQEHFGKDKSK EFQLFSSP-HGKDLL FKDSAHGFLKVPPRM DAKMYLGYEYVTAIR  324
pTf  DdYenCyLAQVPSH. VVARSv.GqED.IWE LLNQAQEHFGrDKS. dFQLFSS.-HGKDLL FKDSAnGFL.iP.kM Ds.lYLGYqYVTAlR  328
pLf  e.freCHLArVPSH. VVARSv.GKEn.IWE LL.QsQkkFGK...q EFQLF.SP...KDLL FrDa..GFLKiP.k. DsKlYLG..YlTAIq  324
sax  nkYKeCnLg.VPa.T VV.R....K.e.I.. .L.eAQk-------r q.kLFSS.-HGKDLm F.DS......l...v DA.lYLG.k...A.k  460
pICA DdYenCyLAQVPSH. VVARSv.GKEDLIWE LLNQAQEnFGKDKS. EFQLFSS.-HGKDLL F.Da..GFLrVPPkM DAKlYLGYEY..AIq  324

                          a'          b'          b'         a'              Δ          h'
hTf  NLREGTCPEAPTDEC K.VKWCALSHHERLK CDEWSVNSVGKIECV SAETTEDCIAKIM.G EADAMSLDGGFVYIA GKCGLVPVLAENYNK  414
pTf  NLRE...Pds..nEC KkVrWCAi.H.E..K CD.WSiNS.GKIECV SAE.TEDCIAKIvKG EADAMSLDGGyiYIA GKCGLVPVLAENY..  415
pLf  .LRE.........--. .kV.WCAv...E..K C.qWS..S...l.C. .A.TTEDCI.qvlKG EADAMSLDGGFiY.A GKCGLVPVLAEN...  412
sax  .L.......s.---- .kVrWC.i.k.EkmK CDdWS..S.G.I.C. .A...k.Cv.qIlKG EADAv.Le..ymY.A ..CGLlP.v.E.hNK  546
pICA hLR.....E.P---- qrV.WCAv..HER.K CD.WSV.S.G.l.C. S.dT.EDCIA.I.KG EADAMSLDGGFlY.A GKCGLVPVLAENY..  410

            i'      Δ                          c'     ↓              j'        d'         e'
hTf  S-------DNCEDTP EAGYFAVAVVKKSAS -DLTWDNLKGKKSCH TAVGRTAGWNIPMGL LYNKINHCRFDEFFS EGCAPGSKKDSSLCK  496
pTf  ..-------eNC.nTP E.GY.AVAVVKKSs. .DL.WnNLKGKKSCH TAV.RTAGWNIPMGL LYNKIN.CkFDqFF. EGCAPGSqrnSSLC.  502
pLf  .....---..C...P ..GYFAVAVVrKa.. -.iTWnsvrG.KSCH TAV.RTAGWNIPMGL L.Nq...CkFDEFFS q.CAPGSq..SnLC.  498
sax  .D...........T. .G...AVAlVKKS.. -Di.WnNiKGKKSCH TgVG..AGW.IPv.L i..q.dn...D.FF. E.CAPGS...SnLCK  635
pICA .D........C.nTP ..GYy.VAVVKKS.a -DLTWnsLrGKKSCH .AVG.sAGW.IPMG. iYNq...Ck.DEFFS q.CAPGS..eS.LC.  499

     d'          e'          Δ      c'                                        f'          f'
hTf  LCMGSG-----LNLC EPNNKEGYYGYTGAF RCLVE-KGDVAFVKH QTVPQNTgGKNPDPW AKNL.EKDyELLCLD GTRKPVEEY.NCHLA  580
pTf  LC.GS.R-A-P...C ..NNhE.YYGYTGAF RCLVE-KGDVAFVK. Q.V.QNTdGKN.D.W AKdLkq.DFELLC.n G.RePVD..eNCHLA  589
pLf  LCvG..q-g--vd.C .PNs.E.YYGYTGAF RCL.E..GDVAFVK. .TV..NTnGqN.e.W Ar.Lr..DFELLCLD GTRKPV.E.qNCHLA  585
sax  LC.G..k.s.....C ..sdKEaYYG..GAF RCLVE-KGDVAFV.H ..V.eNTdGKNP..W AKNLk.eDFELLCLD GsR.PV..YksCkLs  724
pICA LC.GS..-g.P.h.C .PNshEGYhGfsGA. RCLVE-KGDVAFVKH .TV.QNTdGrNPe.W AKdLkqeDFqLLC.D GTRKPV.E.qsCHLA  587

       Δ                                                  i'                          j'
hTf  RAPNHAVVTRKDKEA CVHKILRQQQHLFG. NVTDCSGNFCLFRSE T-KDLLFRDDT.CLA KLHD--rNTYEKYLG -EEYVKAVGNLRKCS  666
pTf  RAPNHAVV.R.DK.. CV.e.L.kQQ..FGR hVTDCS.SFCmFkS. T-KDLLFRDDTqCLA rv.---K.TYE.YLG -.dYi.AVaNLRKCS  674
pLf  .AP.HAVVsRKeK.A .V.qvL..eQ..FGR ...DC...FCLFRSE T-KnLLF.DnTeCLA qL..--K.TYEKYLG -.EYV.AiaNLkqCS  671
sax  ..P..AiVTRee..s .V.rIv..QQ.LyGR ...e.-..F.LF.S. ...nLLF.DnTqCL. ......K...E.Y.G ...Y....G..R...  813
pICA ..P.HAVVsRKDK.. .V.r.L..QQ.LFGR N..e.-..F.LFkS. T-.DLLF.DDTeCLA .L.D--K.TYqKYLG -.EYlqAiaNvRqC.  672

          h'
hTf  TSSLLEACTFRRP-- -------  679
pTf  TS.LLEACTF..... .......  696
pLf  .S.LLEAC.F...-- -------  684
sax  sS.Li.ACT....-- -------  826
pICA .S.LLDACTF...-- -------  685
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FIGURE 4: Alignment of the predicted pICA amino acid sequence with those of hTf, pTf, pLf, and saxiphilin. Identical amino acids are listed in capital letters while conservative substitutions are shown in small letters. The positions of disulfide bonds conserved within the transferrin family are shown as *a*−*g* and *a*′−*j*′. Transferrin iron binding ligands are denoted by Δ, while ↓ marks the position of anion binding residues. The ***** in the sax sequence represents the location of the 144 amino acid insert not included in this alignment. (Genbank accession numbers: hTf, M12530; pTf, X12386; pLf, M81327; sax, U05246.)

the pICA sequence also supports the relationship between pICA and the transferrins since, with a single exception (Stratil et al., 1983), all transferrins are glycoproteins that are glycosylated at asparagine residues (Aisen, 1994). The location, number, and identity of the sugar residues varies, and the carbohydrate plays no known role in the function of hTf (Mason et al., 1993). In addition, the C- and N-terminal halves of all transferrins display a high degree of internal sequence similarity which is believed to be the result of a gene duplication event that occurred prior to the divergence of the different transferrin classes (Aisen, 1994; Crichton & Ward, 1992; Welch, 1992). The two halves correspond to the two lobes observed in X-ray crystal structures, with each lobe or half containing a single iron and anion binding site

(Anderson et al., 1989; Bailey et al., 1988). Alignment of the C-terminal 333 residues of pICA with the remainder of the protein reveals 38% identity between the two halves. This value falls within the range of 33−46% that has been observed for vertebrate transferrins (Aisen, 1994) and is identical to that reported for saxiphilin (Morabito & Moczydlowski, 1994).

Members of the transferrin family are also characterized by the presence of a large number of conserved disulfide bridges (Welch, 1992). Of the 19 such disulfide bonds in hTf, 16 appear to be conserved in pICA (Figure 4). Twelve of these (labeled a−f) are found at similar locations in the N- and C-lobe of the protein. Four others (g−j) are only present in one lobe or the other. The high degree of sequence identity and similarity of pICA to members of the transferrin family coupled with the presence of a majority of the conserved disulfides in transferrin strongly suggests that pICA adopts a tertiary structure similar to that observed for the transferrins, namely, a bilobed structure with a deep cleft in each lobe that, in the transferrins, serves as the site for iron and bicarbonate binding (Baker et al., 1987).

pICA and Transferrin Have Distinct Functions. Despite the similarities in primary sequence between pICA and the members of the transferrin family, the latter do not inhibit the catalytic activity of carbonic anhydrase. The CO_2 hydrase activity of nanomolar concentrations of hCA II, as measured by a pH indicator spectrophotometric assay (Roush & Fierke, 1992), is reduced less than 10% by the addition of 1 μM pTf in either the apo or holo form. Other tested siderophilins (hTf, hLf, and bTf) also do not inhibit hCA II under similar conditions. Therefore, the K_i for inhibition of hCA II by porcine transferrin is >9 μM, at least 10^4-fold greater than the measured K_i for the inhibition of hCA II by pICA (Roush & Fierke, 1992). In addition, hTf, pTf, bTf, and hLf demonstrated no binding to an hCA II−Sepharose column under the conditions employed for either native or recombinant pICA binding (data not shown).

Furthermore, pICA differs from the transferrins in its affinity for iron. For this comparison, both standard 31-element ICP analysis and equilibrium dialysis were used to determine the amount of Fe bound to purified, native pICA. By ICP analysis, purified pICA contains <0.01 mol of Fe/mol of protein. Similarly, purified pICA contains <0.008 mol of Fe^{3+}/mol of pICA as assayed by a ferrozine colorimetric assay. Incubation of 10 μM pICA against an equal volume of 100 μM Fe^{3+}NTA in an equilibrium dialysis chamber for 40 h did not increase the stoichiometry of bound iron. For comparison, after equilibration of apo-hTf under identical conditions, we measured 1.7−2.0 mol of bound Fe^{3+}/mol of protein. Both the ICP and equilibrium dialysis data indicate that the affinity of pICA for Fe^{3+} is drastically reduced compared to the Fe^{3+} association constants of 10^{18}−10^{22} M determined for typical siderophilins under similar conditions [for review, see Welch (1992), chapter 4]. Therefore, pICA does not share the high affinity for Fe^{3+} common to most transferrins, and, in this respect, it is similar to saxiphilin which also shows low affinity for iron despite considerable sequence identity to the transferrins (Li et al., 1993). Of the other 30 elements surveyed by ICP analysis, only significant stoichiometries of Ca^{2+} (0.6 mol/mol of pICA) and Na^+ (1.8 mol/mol of pICA) were observed. Although the high sodium value likely reflects inadequate dialysis of the sample, the nearly stoichiometric amount of calcium may suggest that pICA does possess some affinity for this element.

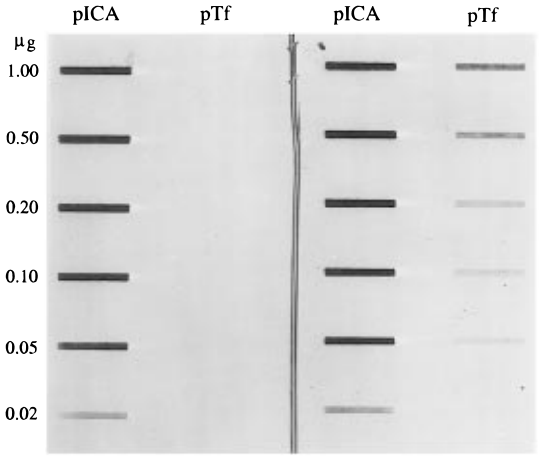

FIGURE 5: Cross-reactivity of rabbit anti-pICA sera with pTf. The stated amount of each protein was directly blotted onto nitrocellulose using a Bio-Rad slot blot apparatus (figures on left apply for all lanes). Primary antibody was either affinity-purified anti-pICA serum (lanes 3 and 4) or the same serum deselected for binding to pTf (lanes 1 and 2).

Avid iron and anion binding, which are a hallmark of the transferrin family, are accomplished by the side chains of 10 invariably conserved residues, as observed in the high-resolution crystal structure of hLf (Anderson et al., 1989). In each lobe, the side chains of one aspartate, one histidine, and two tyrosine residues coordinate the iron while a single arginine interacts with the bound anion. As seen in Figure 4, of the eight transferrin iron ligands, seven are present in the pICA amino acid sequence. The eighth, a tyrosine at position 517 in hTf, has been replaced by a phenylalanine in pICA at position 524. In addition, both of the two arginines used for anion binding in the transferrins have been replaced by Trp124 and Thr459 in the pICA sequence. The loss of these three residues likely accounts for pICA's decreased Fe^{3+} affinity. Woodworth et al. (1991) have shown that, in the N-terminal half-molecule of hTf, replacement of the iron binding ligand Asp63 with a Ser greatly decreases the Fe^{3+} affinity of the ternary Fe^{3+}/anion complex. However, since binding of an Fe^{3+} ion to transferrin is completely dependent upon cobinding of the anion (generally bicarbonate) to the same site (Aisen, 1994), the loss of both anion binding sites in pICA is most likely the major contributor to its inability to bind iron. A number of naturally occurring, nonfunctional iron binding sites in Tfs have been characterized, such as the C-terminal sites of melanotransferrin (Baker et al., 1992; Rose et al., 1986) and the *Manducta sexta* serum Tf (Bartfeld & Law, 1990) and both sites of saxiphilin (Morabito & Moczydlowski, 1994). The N-terminal site of pICA represents the first instance of a site where loss of the anion binding residue alone (Arg → Trp124) significantly decreases iron binding.

Antigenic Relationship between pICA and Transferrin. Given the similarities in primary sequence between pICA and the transferrins and their differences in apparent function, we decided to test whether pICA and the transferrins share antigenic determinants. An antiserum to pICA was affinity-purified and then tested against other members of the transferrin family in a slot-blot assay. As shown in Figure 5 (lanes 3 and 4), pTf is recognized by the anti-pICA serum at a concentration that is approximately 250-fold higher than

that required for observing pICA. Other tested transferrins were either recognized even less efficiently (human Lf) or not recognized at all (bovine Tf and hTf), and preimmune serum did not react with pICA or any other tested siderophilin (data not shown). Although preincubation of the anti-pICA sera with pTf before screening does not alter the ability of the serum to recognize pICA, as seen in Figure 5 (lanes 1 and 2), virtually all antibody binding to pTf is lost. This suggests that while pICA shares some antigenic determinants with porcine transferrin, the major antigenic determinants of pICA differ significantly from those of most transferrins. Again, in this respect, pICA is similar to saxiphilin (Li et al., 1993).

Function of Transferrin-Related Proteins. The determination of the sequence of the porcine gene encoding a carbonic anhydrase inhibitor that is secreted into plasma indicates that this protein has significant sequence identity with members of the transferrin family, including conserved disulfide bonds and glycosylation. On the basis of this high degree of similarity, it appears that pICA arose from a two-lobed transferrin ancestor. Subsequent amino acid substitutions in the anion and iron binding sites significantly decreased the affinity of pICA for iron such that the apoprotein accumulates in plasma and minimal iron binding is detected under equilibrium dialysis conditions. Furthermore, pICA has gained a high-affinity binding site for specific carbonic anhydrase isozymes that also inhibits the CA activity. Saxiphilin is a "transferrin-related protein" that has lost the capacity to bind iron and gained a new binding site for saxitoxin (Li et al., 1993; Morabito & Moczydlowski, 1994). These two proteins suggest the existence of a superfamily of transferrin-related proteins where the tertiary fold of transferrin is adapted to bind a wide range of both small and large ligands. In fact, the broad variety of additional functions attributed to lactoferrin including activation of natural killer cells, granulopoiesis, cytokine production, and cell growth [for review, see Iyer and Lonnerdal (1993)], may be due to a family of related proteins with altered sequences and binding specificities, including affinity for specific DNA sequences (He & Furmanski, 1995). The proposed binding site of saxitoxin in saxiphilin is located in an interdomain cleft analogous to the location of one of the homologous Fe^{3+}-binding sites of transferrin (Llewellyn & Moczydlowski, 1994). Although we have not definitively identified the CA II binding site in pICA, preliminary proteolysis experiments suggest that it resides in the final 270 amino acids of the C-terminal domain (E. D. Roush, S. Siang, and C. A. Fierke, unpublished results).

The available data suggest that the main role of pICA in plasma is to form a complex with CA II which has two consequences: (1) any CA II protein that is leaked into plasma has no catalytic activity and (2) CA II is no longer filtered from plasma at the kidney glomeruli but is preferentially localized to the liver (Appelgren et al., 1989; Ojteg & Wistrand, 1994). A similar inhibitor has been observed in many mammals (Booth, 1938; Hill, 1986; Leiner et al., 1962; Rispens et al., 1985), but so far there is no evidence that a homolog exists in humans (Booth, 1938; Hill, 1986; Leiner et al., 1962; Rispens et al., 1985; M. W. Wuebbens and C. A. Fierke, unpublished data). A number of other plasma proteins have been identified that bind red blood cell components, including hemopexin, which binds free heme, and haptoglobin, which binds hemoglobin (Muller-Eberhard, 1988; Putnam, 1975). Both hemopexin and haptoglobin are present in micromolar concentrations in the plasma and are bound by specific receptors in the liver to prevent excretion of heme and hemoglobin via the kidneys (Kino et al., 1980; Smith & Hunt, 1990). Several of the proposed roles of these proteins are to protect against the oxidative effects of free heme and to recycle heme for iron conservation and regulation of iron and heme metabolism (Halliwell & Gutteridge, 1990; Muller-Eberhard & Vincent, 1985; Vincent et al., 1988). Similarly, the main roles of pICA could be to recycle CA II and its bound zinc via uptake by the liver and/or to inhibit the hydrolytic activity of CA II in plasma, perhaps to facilitate the Bohr effect.

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