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Mammalian Tyrosinase-Related Protein-1 is Recognized by Autoantibodies from Vitiliginous Smyth Chickens

An Avian Model for Human Vitiligo

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The Smyth line (SL) chicken is an animal model for the human acquired depigmentary disorder vitiligo. Affected birds from this line express a postnatal loss of melanocytes in feather and ocular tissues. This vitiligo-like depigmentation is considered to be a disorder with two interacting components: melanocyte dysfunctions and autoimmune reactions. Previously, SL chicks were shown to express high levels of circulating autoantibodies that bind to chicken melanocyte proteins with molecular masses between 65 and 80 kd. Three mammalian melanocyte proteins known to have isoforms in this molecular mass range are tyrosinase, tyrosinase-related protein (TRP)-1 and TRP-2. Of these, only tyrosinase is reported to be expressed in chicken melanocytes. The results presented in this study indicate that, of these three candidate proteins, TRP-1 is the primary antigen recognized by the SL autoantibodies. SL autoantibodies recognize a chicken melanocyte protein that is different from that of tyrosinase or the candidate chicken TRP-2. In addition, several types of experiments incriminate TRP-1 as the primary mammalian melanocyte antigen recognized by SL autoantibodies. We further verified that chicken melanocytes expressed messages for TRP-1 by finding positive signals on Northern blots of chicken melanocyte RNA probed with mammalian TRP-1 cDNA fragments. Therefore, we conclude from these results that the SL autoantibodies primarily recognize TRP-1 in mammalian melanocytes and suggest that chicken melanocytes express a homologue of TRP-1 (the human gp75 and the murine* brown/b *locus protein). (Am J Pathol 1995, 146:1529–1541)

Vitiligo is an acquired cutaneous depigmentary disorder of humans that presents with focal lesions, devoid of melanocytes, which can expand to encompass most of the body surface (for review see Ref. 1). Patients expressing vitiligo can also present with visual impairment and associated disorders that appear to be autoimmune in nature, eg, thyroiditis, alopecia areata, pernicious anemia, and diabetes. The etiology of vitiligo is not clearly defined, although pathological mechanisms involving inherent melanocyte dysfunction, autoimmune response, and/or cytotoxicity to environmental agents have been proposed.

Smyth line (SL) chickens express a genetically inherited form of post-hatch human vitiligo-like depigmentation (reviewed by Smyth[2]). These chickens have been studied as one of the animal models for the human disease vitiligo.[1,3] The vitiliginous phenotype in the chicken model appears to result from the interaction of aberrant melanocytes[4] and autoimmune reactions mediated by both B and T cell components.[4–7] Recently, circulating autoantibodies were detected in 100% of the vitiliginous SL chicks.[8] These SL autoantibodies recognized up to three bands in the 65- to 80-kd range on immunoblots of chicken melanocyte lysates. Although it is not known whether these autoantibodies are involved in the autoimmune response, the identification of the autoantigen may provide an important clue in determining how the aberrant melanocytes may be interacting with the immune system.

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There are three known mammalian melanocyte proteins reported to have isoforms within the 65- to 80-kd molecular mass range. These are tyrosinase (TYR), tyrosinase-related protein (TRP)-1 (human gp75, mouse *brown* locus), and TRP-2 (the enzyme dopachrome tautomerase, mouse *slaty* locus).[9]

The multifunctional enzyme TYR (monophenolmonooxigenase; EC 1.14.18.1) has been reported to be the one primary enzyme essential for melanogenesis. In the search for a TYR cDNA, the sequences for three additional melanocyte proteins were discovered. An anti-TYR antibody (Ty)[10] identified three additional melanocyte proteins. Two of these three proteins, TRP-1[11] and TRP-2,[12] have been observed to have isoforms in the 65- to 80-kd molecular mass range. Each is encoded on separate chromosomes in humans and mice yet have regions of strikingly similar amino acid homology that may explain their antigenic cross-reactivity. Collectively TYR, TRP-1, and TRP-2 are considered to be members of a multigene family of TRPs.[9]

Of the three candidate proteins, TYR is the only one reported in the literature to be expressed by chicken melanocytes. Chicken TYR from albino, white, black, and Light Brown Leghorn chickens was isolated and characterized as a 66-, 68-, 72-, or 82-kd protein, respectively, with multiple isoforms.[13,14] On the basis of biochemistry studies comparing newly synthesized proteins between melanin-producing and non-melanin-producing chicken melanocytes, at least nine protein groups have been hypothesized to be involved in chicken melanogenesis.[14] We initiated this study to identify the primary melanocyte antigen(s) recognized by the SL autoantibodies and to determine whether the antigen is one or more of the following melanocyte proteins: TYR, TRP-1, TRP-2, and/or a novel protein. Although TRP-1 and TRP-2 have not been reported to be expressed by avian melanocytes, in the course of this study we found evidence that chicken melanocytes express homologues of mammalian TRP-1 and TRP-2.

Materials and Methods

Melanocyte Antibodies

A description of the antibodies and their sources are given in Table 1.

Cell Lines and Sources

Chicken melanocytes,[15] chicken fibroblasts,[8] murine melanocytes,[16] mouse fibroblasts, murine B-16 melanoma cells, normal human melanocytes from neonatal foreskins,[17] normal human melanocytes from adult punch biopsies,[18] human melanoma cells (IIB-MEL-J),[19] human fibroblasts, Cloudman S91 murine melanoma cells (DBA × C) F_1,[20] and N & S-type human neuroblastoma cells (a gift from J. Foley and L. Parysek of the University of Cincinnati)[21] are routinely cultured in our laboratory.

Immunoblotting

The immunoblotting procedure has been described previously.[8] Semiconfluent melanocytes were collected from the flasks, rinsed twice in phosphate-buffered saline (PBS) and solubilized in 10 mmol/L Tris buffer (pH 8.0) with 1 mmol/L phenylmethylsulfonyl fluoride, 20 μmol/L leupeptin, 15 μmol/L pepstatin, 50 mmol/L *o*-phenanthroline, and 20 μmol/L benzamidine with 1% sodium dodecyl sulfate (SDS) for 0.5 hour at 4 C. After centrifugation to remove the unsolubilized material, the lysates were separated on SDS-polyacrylamide gel electrophoresis (PAGE) reducing gels and electroblotted onto nitrocellulose. The blotted proteins were blocked in 5% Carnation non-fat instant dry milk with 100 mmol/L NaCl, 50 mmol/L Tris, pH 7.5, and 0.05% Tween 20. The blots were incubated with the primary antibody (see figure legends for dilutions) for 3 hours at room temperature,

Table 1. *Melanocyte Antibodies and Antigenic Sources*

Antibody	Antigen	Antibody type
PEP1[22]	C-terminal peptides of mouse TRP-1	Polyclonal
PEP2[22]	N-terminal peptides of mouse TRP-1	Polyclonal
PEP5[32]	N-terminal peptides of mouse TYR	Polyclonal
PEP7[32]	C-terminal peptides of mouse TYR	Polyclonal
PEP8[25]	C-terminal peptides of mouse TRP-2 (DOPAchrome tautomerase)	Polyclonal
Ty[10]	Hamster melanoma proteins including TYR	Polyclonal
BF[47]	Human TYR	Polyclonal
TA99[39]	Human melanoma TRP-1 (*gp75*)	Monoclonal
SL[8]	? Vitiliginous	Polyclonal (?)
TMH-1[48]	Mouse TRP-1	Monoclonal

rinsed three times, 10 minutes each, and then incubated in the corresponding alkaline phosphatase-conjugated secondary (1:1000 dilution; goat anti-chicken for SL autoantibodies and goat anti-rabbit for PEP and Ty antibodies). The blots were developed with the premixed 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (BCIP/NBT) phosphatase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

Radiolabeling/Immunoprecipitation of Native Proteins

The basic radiolabeling and immunoprecipitation procedure is described in detail by Austin et al.[8] Cells were plated into 100-mm plastic dishes at approximately 1×10^6 to 2×10^6 cells per dish. After 1 to 2 days, cells were rinsed three times at 37 C with PBS and incubated in serum-free Eagle's minimal essential medium (MEM) without cysteine-methionine, with 1% L-glutamine added, for approximately 2 hours. The cells were then pulsed for 3 to 5 hours with 50 to 100 µCi/ml [^{35}S]cysteine-methionine in Eagle's MEM. The cells were rinsed three times (the chicken melanocytes were rinsed once) with cold PBS and then lysed in the dish in 1 ml of lysis buffer consisting of 50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1% NP-40, 4 mmol/L diisopropyl fluorophosphate, 20 mmol/L leupeptin, 15 mmol/L pepstatin, 30 mmol/L E-64, and 50 mmol/L 1,10 phenanthroline at 4 C. The lysates were centrifuged, and 5 µl of the lysate was precipitated in 10% trichloroacetic acid to establish dpm levels for the immunoprecipitation experiments. Samples were immunoprecipitated with 10 µl of the primary antibody (SL and PEPs) or 2 µl of the TA99 hybridoma supernatant. Immunoprecipitations with chicken serum included 2 µl (1:40 dilution) of rabbit anti-chicken immunoglobulin G as a bridging antibody; TMH-1 immunoprecipitations included 2 µl (1:40 dilution) of rabbit anti-rat antibody. This was followed by incubation at 4 C with 40 µl (10%) of protein A-Sepharose. The protein A complexes were rinsed three times in lysis buffer, the pellets were resuspended in 2X sample buffer (0.125 mol/L Tris-Cl, pH 6.8, 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol) and heated at 85 C for 10 minutes. The protein A was again pelleted, and 2 µl of supernatant were removed and counted with a Packard TriCarb 1900 CA liquid scintillation analyzer. An approximately equal number of dpm per lane for each gel (which could vary from 500 to 2000 dpm per gel depending on the efficiency of labeling and the experiment) and separated by 8% SDS-PAGE. The gels were processed for fluorography with two 30-minute incubations in 20% 2,5-diphenyl oxazole in dimethylsulfoxide followed by two 30-minute rinses in distilled water. The gels were dried and exposed on Kodak X-OMAT film.

Immunoprecipitation of Denatured Proteins

The melanocytes were metabolically labeled and collected as described above, and the proteins were then denatured with the procedure described by Jiménez et al.[22] The cell pellet was incubated in 200 µl of 1% SDS with 2 mmol/L dithiothreitol at 70 C for 5 minutes. This was followed by the addition of the lysis buffer described above, which also contained 20 mmol/L iodoacetic acid. Immunoprecipitation was done as described above, and 5×10^6 dpm were immunoprecipitated with 10 µl of the PEP antibodies and 10 µl of the SL serum.

Protein Trafficking

Biosynthesis and glycosylation rates were determined by the following methods. Briefly, cells were prepared as described above, with the medium containing 100 to 200 µCi/ml [^{35}S]cysteine-methionine in Eagle's MEM. The cells were radiolabeled for 10 minutes followed by chases of specified times in normal growth medium. The samples were collected and processed as described above. After rinsing the pellets with lysis buffer, samples were incubated at 100 C for 5 minutes in 15 µl of 0.5% SDS, 0.1 mol/L sodium citrate, pH 6.0, followed by the addition of 30 µl of 1.25 mmol/L EDTA, 1 µmol/L phenylmethylsulfonyl fluoride, and 0.1 mol/L sodium citrate, pH 6.0. The solution was divided into two, one for recombinant endo-B-*N*-acetylglucosaminidase H (Endo-H) digestion and one for a mock digestion, without Endo-H. A 30-ng volume of Endo-H diluted in the sodium citrate buffer, was added per 45 µl of sample with an equal amount of the sodium citrate buffer added to the mock digestions. Digestions were at 37 C for 18 to 20 hours. Pellets were resuspended in 3X sample buffer, heated at 85 C for 10 minutes, and further processed as described above.

Tyrosinase Activity after Immunodepletion

Briefly, 5×10^6 Light Brown Leghorn melanocytes were processed as described above for the radiolabelling procedure. Briefly, 220 µl of supernatant was removed for each evaluation of tyrosine hydroxylase activity (assayed in duplicate, 100 µl per sample). A

baseline level of tyrosine hydroxylase activity was established before the preclearing step. The supernatants (cell lysates) were divided into nine tubes, brought to 1 ml with lysis buffer, and then precleared with 40 μl of Protein A-Sepharose (10%) for 1 hour at 4 C followed by the removal of another 220 μl of supernatant to determine the background removal by the protein A-sepharose. The supernatants were incubated for three successive immunodepletions with one of the following: 20 μl of SL autoantibodies from vitiliginous or nonvitiliginous SL chickens (1:2 dilution), previously determined to be positive or negative, respectively, for SL melanocyte autoantibodies, 20 μl of wild-type Light Brown Leghorn serum (1:2 dilution), 10 μl of Ty (anti-hamster tyrosinase) rabbit antibody (positive control), or 10 μl of normal rabbit serum (negative control). After each primary antibody incubation, supernatants were incubated with 40 μl of 10% protein A-Sepharose followed by the removal of another 220 μl of supernatant. The TYR assay on the supernatants was performed with [^{3}H]tyrosine (NEN Research Products, Boston, MA).[15] The remaining tyrosine hydroxylase activity in the depleted lysates, between the vitiliginous and nonvitiliginous SL serum, was compared by Student's *t*-test.

Northern Blot Analysis

Total RNA was isolated by either the Chomczynski technique[23] or cesium chloride gradients (Maniatis et al[24]). mRNA was isolated by oligo dT columns.[24] The RNA was separated on 1.2% agarose/ethidium bromide formaldehyde gels in 1X 3-[*N*-morpholino]propanesulfonic acid. After separation, the RNA was transferred to nylon membranes and stored at −85 C. The human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA was purchased from Clontech Laboratories (Palo Alto, CA). The TRP probes were generated by isolating the TRP cDNA fragments from the plasmids described in Table 2. These probes were labeled by random priming with [^{32}P]ATP by the Megaprime DNA labeling system (Amersham, Arlington Heights, IL). The membranes were prehybridized for 3 to 10 hours in the hybridization buffer consisting of 10% dextran sulfate sodium salt, 40% formamide, 5X standard saline citrate (SSC), 5X Denhardt's solution, 100 μg/ml DNA sodium salt type XIV: herring testes, and 0.1% SDS. The entire probe solution was added directly to the hybridization solution and incubated for 12 to 15 hours at 42 C. The membranes were rinsed twice with 2X SSC buffer at 42 C followed by several rinses with 0.5X SSC at 55 C. Several rinses with 0.1X SSC buffer at 55 C were included when necessary to reduce background. Images were obtained with autoradiographic film.

Table 2. *Description of TRP Probes for Northern Analysis*

cDNA	Species	Size	Source	Reference
TRP-1	Mouse (B^{lt})	1.1 kb	I. J. Jackson	
TRP-1	Human	2.7 kb	S. Vijayasaradhi	
TRP-2	Mouse	1.75 kb	I. J. Jackson	12
TYR	Human	1.8 kb	W. Oetting	49

Results

Smyth Line Autoantibodies Recognize Chicken Melanocyte Proteins with Different Molecular Weights from TYR or TRP-2

Several isoforms of three mammalian melanocyte proteins, TYR, TRP-1, and TRP-2, have been reported (and/or observed) to have molecular masses between 65 and 80 kd. The first step of this study was to determine the relative molecular masses of TYR, TRP-1, and TRP-2 in chicken melanocytes (see Table 1 for a description of the antibodies). Lysates of cultured wild-type Light Brown Leghorn and SL melanocytes were immunoprecipitated with antibodies that recognize human TYR (BF), the carboxyl-terminal region of mouse TRP-2 (PEP8) and the SL serum. A potential chicken TYR protein with a molecular mass of approximately 82 kd, a greater molecular mass than the SL antigen(s), was identified with the BF antibody (Figure 1A). We also tested the anti-mouse TYR antibody PEP7 on immunoblots of chicken melanocyte proteins and found that this anti-TYR antibody also recognized a protein of higher molecular mass than that of the SL autoantigen (Figure 1B). In addition, we tested for SL autoantibody depletion of tyrosine hydroxylase activity, indicative of TYR (see the experiment described below in Figure 2). Therefore, we believe that TYR is not a primary antigen recognized by SL autoantibodies.

We then ruled out TRP-2 as a primary antigen for the following reason. TRP-2 has two distinct isoforms, as recognized by the PEP8 antibody in mouse melanocytes,[25] that is similar to the pattern shown in Figure 1A by chicken melanocytes. These isoforms are either above or below the protein(s) recognized by the SL autoantibodies (Figure 1A).

We then continued to explore TRP-1 as a candidate antigen by immunoprecipitations and immunoblots with anti-mouse or anti-human TRP-1 antibodies. We found that neither the monoclonal anti-human TRP-1

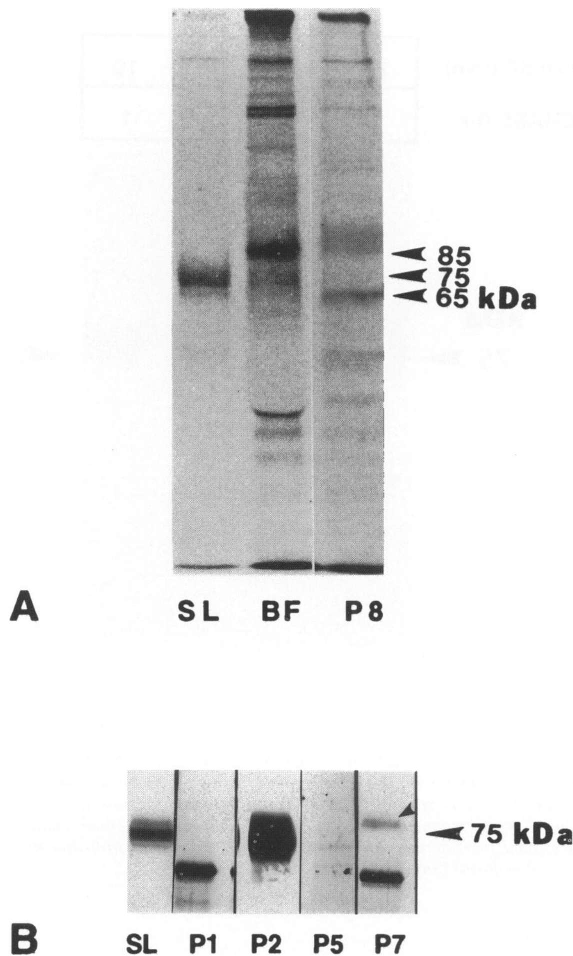

Figure 1. *SL autoantibodies do not appear to recognize chicken TYR or chicken TRP-2.* **A:** *Wild-type chicken melanocytes were metabolically labeled with [^{35}S]cysteine/methionine, solubilized with 1% Triton X-100, divided into samples containing 1 × 10^{6} dpm, and immunoprecipitated with the following antibodies: 20 μl of SL serum (1:2), 5 μl of anti-human TYR (BF), and 10 μl of anti-mouse TRP-2 PEP8 (P8).* **B:** *SL melanocytes were lysed, separated on denaturing SDS-PAGE, and blotted. These blots were probed with the following antibodies: SL (1:1000), anti-mouse TRP-1 antibodies PEP1 and PEP2 (1:500), and anti-mouse TYR antibodies PEP5 and PEP7 (1:500).*

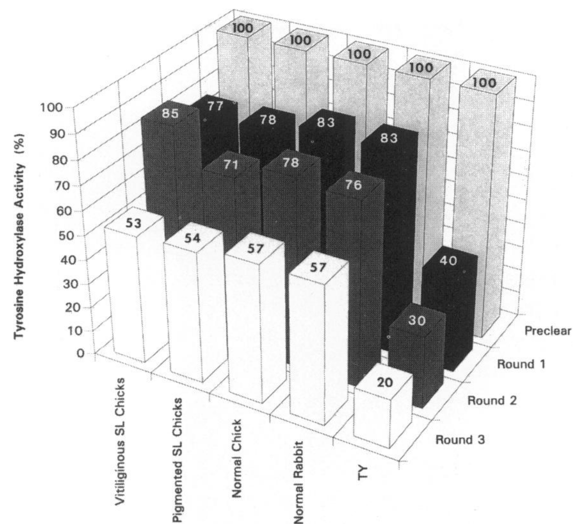

Figure 2. *SL autoantibodies do not remove tyrosine hydroxylase activity. Chicken melanocyte lysates were divided into nine aliquots, precleared by immunoprecipitation with 40 μl of 10% protein A-Sepharose followed by three rounds of immunoprecipitation with 20 μl of serum from vitiliginous SL chicks (1:2 dilution; n = 4 for rounds 1 and 3; n = 3 for round 2), pigmented SL chicks (1:2 dilution; n = 2), and a wild-type chick (1:2 dilution; n = 1) of similar age. For comparison, 10 μl of rabbit anti-TYR antibodies (Ty) (n = 1) and normal rabbit serum (n = 1) were also tested. After each round of immunoprecipitation, two samples of 100 μl each of lysates were tested for tyrosine hydroxylase activity.*

antibody TA99 nor the polyclonal anti-mouse TRP-1 antibody PEP1 (which recognizes the carboxyl-terminal region of TRP-1) immunoprecipitated candidate TRP-1 proteins from radiolabeled chicken melanocyte lysates (results not shown). Also, PEP1 does not recognize a candidate TRP-1 protein on immunoblots of chicken melanocyte lysates (Figure 1B). We then tested the anti-mouse TRP-1 antibody PEP2, which recognizes only denatured TRP-1, on denatured chicken melanocyte lysates both by immunoblotting and by immunoprecipitation. We found that, although PEP2 recognized a protein of the same molecular mass as the protein recognized by the SL autoantibodies on immunoblots, it appeared as if PEP2 was also recognizing an additional higher molecular mass protein from SL melanocyte lysates (Figure 1B). We then immunoprecipitated a potential TRP-1 protein with PEP2 from denatured chicken melanocyte lysates that had the same molecular mass as the antigen recognized by the SL autoantibodies (Figure 3A). No additional proteins were immunoprecipitated by PEP2. We then designed several experiments to verify whether TRP-1 was the primary antigen recognized by the SL autoantibodies.

Smyth Line Autoantibodies Do Not Recognize Chicken Tyrosinase

The data presented in the immunoprecipitation experiments described above indicated that the SL antigen was not TYR. To confirm this observation, we performed immunodepletion experiments with the SL autoantibodies to determine whether these autoantibodies removed tyrosine hydroxylase activity from chicken melanocyte lysates, an indication that the antigen was TYR. After preclearing, the chicken melanocyte lysates were subjected to three successive rounds of immunoprecipitation with several types of antibodies. After each round, tyrosine hydroxylase activity was measured. As shown in Figure 2, the anti-TYR antibody (Ty), which recognizes mammalian and

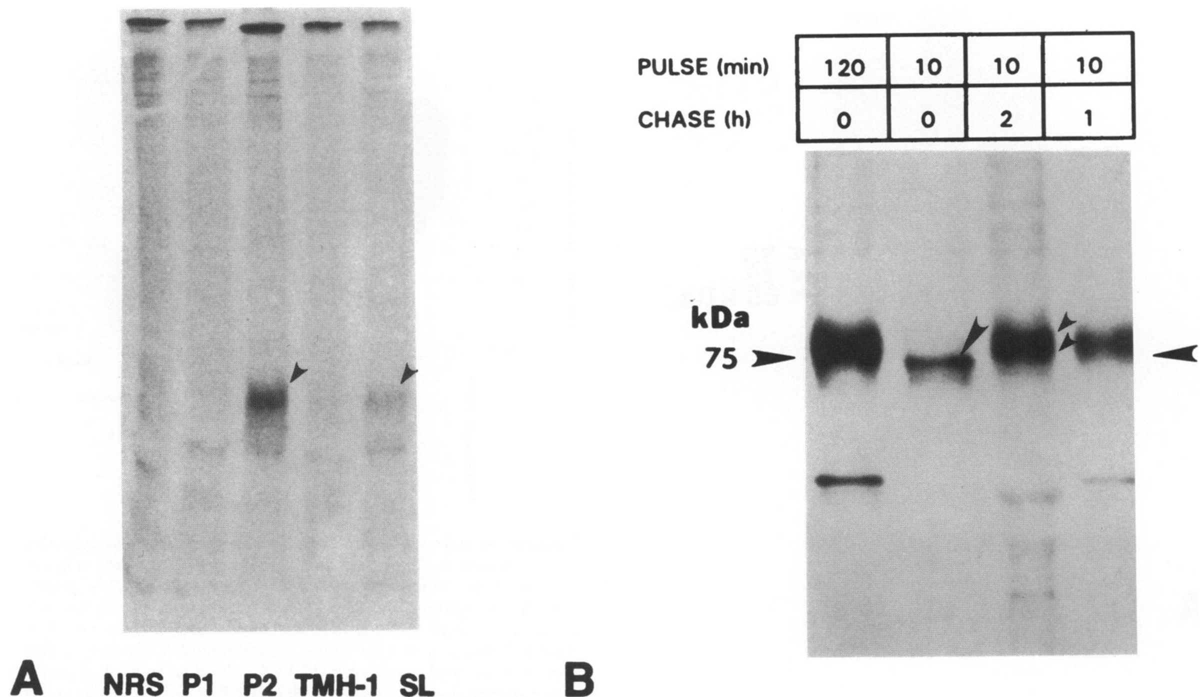

Figure 3. *SL autoantibodies recognize a chicken melanocyte protein that could be TRP-1.* A: *The PEP2 anti-mouse TRP-1 antibody recognizes a SL melanocyte protein of the same molecular mass as the SL autoantigen. The melanocytes were radiolabeled for 4 hours, the cells were lysed in 200 µl of buffer, and incubated for 5 minutes at 70 C. A 10-µl volume of each antibody source, normal rabbit serum (NRS), PEP1 (P1), PEP2 (P2), and SL, and 35 µl of TMH-1 were added to 5 × 10⁶ dpm of trichloroacetic acid-precipitable sample.* B: *The protein identified by the SL autoantibodies in Light Brown Leghorn melanocytes have a similar rate of synthesis, number, and molecular mass of isoforms as that reported for human TRP-1 in melanoma cells. The melanocytes were pulsed for 10 minutes with 200 µCi/ml [³⁵S]cysteine/methionine and chased with regular growth media for the times indicated. Cells were processed for SDS-PAGE fluorography as described in Materials and Methods. Large arrowhead indicates the lower molecular mass precursor, and the small arrowheads indicate the subsequent isoforms (products).*

chicken TYR, removed significant amounts of tyrosine hydroxylase activity from chicken melanocyte lysates.

In contrast, chicken melanocyte lysates immunoprecipitated with either normal chicken (n = 1), vitiliginous SL (n = 4), or nonvitiliginous (pigmented) SL (n = 2) serum, as well as normal rabbit serum (n = 1), exhibited similar losses in tyrosine hydroxylase activity (Figure 2). There were no significant differences ($P = 0.001$) between the tyrosine hydroxylase activity remaining in lysates immunodepleted with serum from vitiliginous and nonvitiliginous SL chickens after the third round of immunoprecipitation. The vitiliginous and nonvitiliginous chicken serum had previously been shown to be positive and negative, respectively, for SL autoantibodies on immunoblots of chicken melanocyte proteins. Therefore, on the basis of these results and the results of the immunoprecipitation experiments described in the previous section, it was concluded that the SL autoantibodies do not specifically recognize TYR or a protein with tyrosine hydroxylase activity, as detected by this assay.

Smyth Line Autoantibodies Appear to Recognize One Chicken Melanocyte Protein with Two Primary Isoforms that Is a Potential TRP-1 Candidate

Pulse/chase ^{35}S-radiolabeling of chicken melanocytes followed by immunoprecipitation with SL autoantibodies, repeated with serum from two different white SL chicks, demonstrated that the lower molecular mass protein was synthesized within the first 10 minutes of the pulse, and the two higher molecular mass proteins appeared during the 1 hour chase (Figure 3B). These data suggest that the primary SL melanocyte autoantigens[8] are one protein (precursor) that was processed into two primary isoforms (products) within 1 hour of synthesis. These results are similar to those obtained by the trafficking experiments of human melanoma TRP-1 by Vijayasaradhi et al[26] in which the TRP-1 monoclonal antibody TA99 recognized a protein of 58 kd after 10 minutes of pulse, which was then processed into two higher molecular mass isoforms with 30 minutes of chase.

Smyth Line Autoantibodies Recognize a Human Melanocyte Protein that Correlates with Human TRP-1

As the rate of synthesis and of glycosylation (ie, trafficking) of human TRP-1, as identified by the monoclonal antibody TA99, in human melanoma cells has been reported,[26] we directly compared the trafficking of human TRP-1 (with TA99) and the SL antigen. The trafficking of human TRP-2 was also followed as a negative control. In this series of radiolabeled pulse/chase time points, we followed the movement of proteins from the rough endoplasmic reticulum through the Golgi by observing the change in glycosylation patterns and number of isoforms, before and after Endo-H digestion. We found, in side-by-side comparisons, that the trafficking of the SL antigen matched that of human melanocyte TRP-1 and differed from that of TRP-2. The protein recognized by the SL autoantibodies has an identical molecular mass, rate of synthesis, and processing and isoform pattern both before and after Endo-H digestion as human melanocyte TRP-1 (Figure 4A, B). Although we also found many characteristics that were similar for both TRP-1 and TRP-2 (Figure 4C), key differences distinguished TRP-2 from the TRP-1/SL antigen. Specifically, before Endo-H digestion (M lanes), TRP-1 and the SL antigen are beginning to be processed to higher molecular mass forms after just 15 minutes of the chase period (Figure 4A, B). In contrast, TRP-2

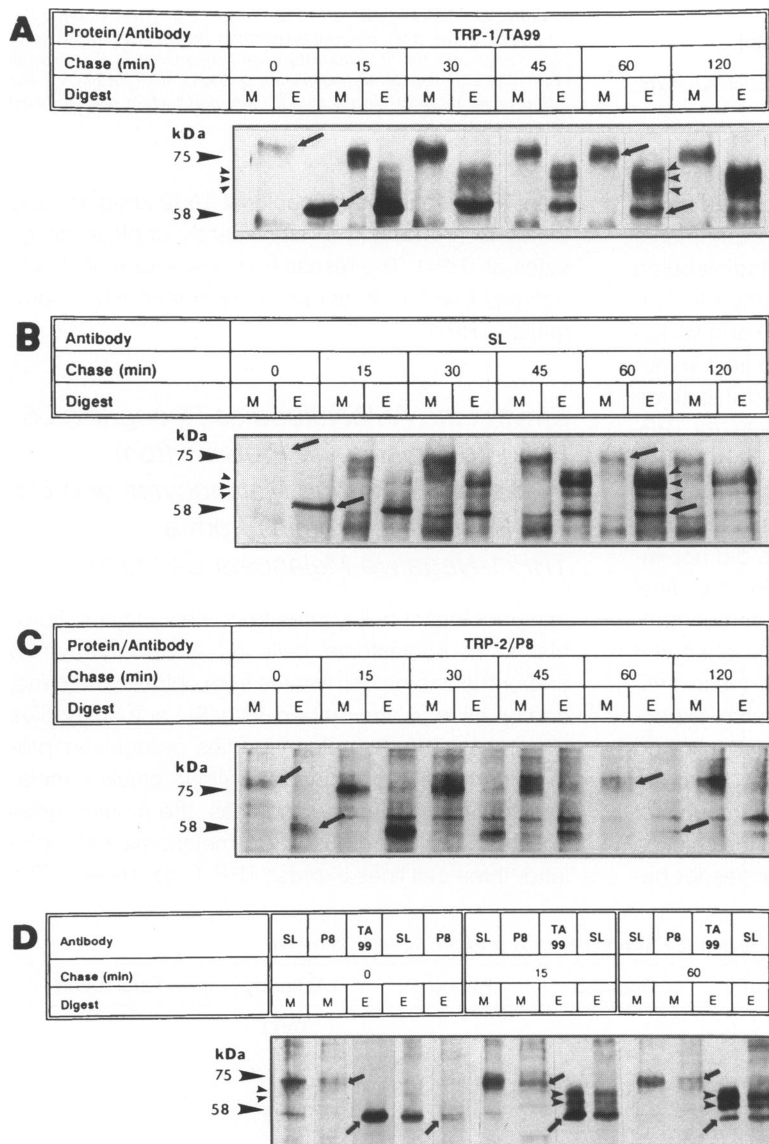

Figure 4. *SL autoantibodies primarily recognize TRP-1 isoforms. Normal human melanocytes were radiolabeled with [^{35}S]methionine/cysteine for 10 minutes and chased with cold medium for the times indicated. The lysates were immunoprecipitated with 2 µl of TA99* (**A**)*, 20 µl of SL autoantibodies* (**B**) *or 10 µl of PEP8 P8* (**C**) *followed by enzymatic digestion of the precipitates with Endo-H (E) or mock digestion without Endo-H (M). Selected comparisons are shown in* (**D**)*. The trafficking pattern of the SL antigen matches that of TRP-1 and not TRP-2. Large arrows indicate the specific undigested protein in the M lane and the specific unprocessed Endo-H-digested protein in the E lane. Small arrowheads indicate the processed isoforms after Endo-H digestion.*

does not progress to a higher molecular mass until after 30 minutes of the chase period (Figure 4C). Within 30 minutes of synthesis, TRP-1 and the SL antigen appear to have reached their higher molecular mass forms. TRP-2 was not observed to be processed into its higher molecular mass form until the 2-hour time point. TRP-1 and the SL antigen have isoforms of identical molecular masses (Figure 4A, B), clearly seen when these products were placed next to each other on a SDS-PAGE gel (Figure 4D). TRP-2 appears to have only one isoform after Endo-H digestion. This isoform does not appear to change over the duration of the chase period (Figure 4C). This experiment is consistent with our hypothesis that the primary antigen recognized by the SL autoantibodies may be TRP-1.

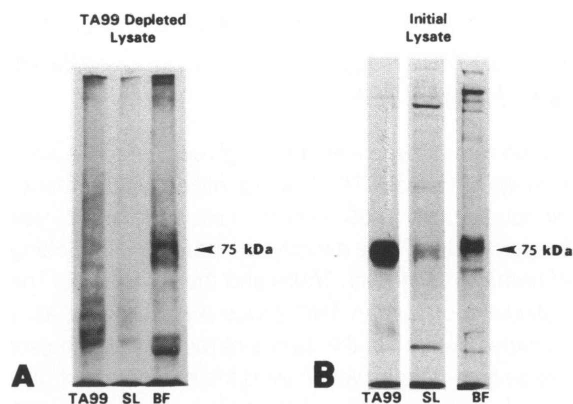

Figure 5. *SL autoantibodies do not recognize proteins in human melanocyte lysates depleted of TRP-1. Normal human melanocytes were radiolabeled with [^{35}S]methionine/cysteine, divided into equal aliquots (1 × 10^6 dpm), and initially depleted with TA99 (10 µl) or SL serum (20 µl). After the fourth round, each sample was divided into three additional aliquots and immunoprecipitated a fifth and final time with TA99 (5 µl), SL (10 µl), or BF (2 µl). The resulting fluorographs of the final immunoprecipitates demonstrates absence of both TRP-1 and the SL autoantigen and the presence of tyrosinase (BF) after complete immunodepletion of TRP-1 with TA99* (**A**). *The immunoprecipitates from the initial immunodepletion are presented for comparison* (**B**).

Smyth Line Autoantibodies Do Not Recognize Proteins in Human Melanocyte Lysates Depleted of TRP-1

To determine whether depleting melanocyte lysates of TRP-1 would remove the protein recognized by SL autoantibodies, we did the following experiments (see Table 3 for experimental design). Radiolabeled normal human melanocytes were subjected to four rounds of immunoprecipitation with TA99 and vitiliginous SL autoantibodies. For the fifth and final immunoprecipitation, each lysate was divided into three aliquots and immunoprecipitated with TA99 (for TRP-1), BF (for TYR), and SL autoantibodies (see Table 3 for a summary of results). When normal human melanocyte lysates were immunodepleted with the monoclonal antibody TA99, SL autoantibodies did not immunoprecipitate a specific protein in the final immunoprecipitation (Figure 5A). These immunoprecipitates can be compared with what is observed after the initial immunoprecipitation with these antibodies (Figure 5B). However, the converse experiment, immunodepleting with SL antibodies followed by immunoprecipitation with TA99, showed that the SL antibodies did not reduce detectable amounts of TA99 binding (Table 3). It seems likely that the chicken autoantibodies have relatively lower affinities for human TRP-1 than the monoclonal TA99 antibody and therefore are unable to significantly deplete the lysates of TRP-1. The results from this experiment also suggest that the SL autoantibodies recognize mammalian TRP-1.

Smyth Line Autoantibodies Recognize 65 to 80-kd Melanocyte Proteins from Humans and Murine Melanocytes and Do Not Recognize Proteins from a TRP-1-Negative Melanoma Cell Line

Immunoblots of cell lysates from melanocytes, fibroblasts, neuroblastoma cells (N and S subtypes) and/or melanoma cell lysates from chicken, humans, and/or mice, were probed with SL autoantibodies (Figure 6). The SL autoantibodies recognized proteins from the pigmented and albino chicken melanocytes, the human melanocytes, the mouse melanocytes, and the mouse S91 melanoma cells (the latter three cell lines express TRP-1, not shown). The

Table 3. *Immunodepletion Experiment Showing the Antibodies Used and the Results Obtained*

Antibody	Round 1	Round 2	Round 3	Round 4	Antibody	Final round
TA99	+	+	+	−	TA99	−
					SL	−
					BF	+
SL	+	+	+	+	SL	−
					TA99	+
					BF	+

+ immunoprecipitate present; − no immunoprecipitate.

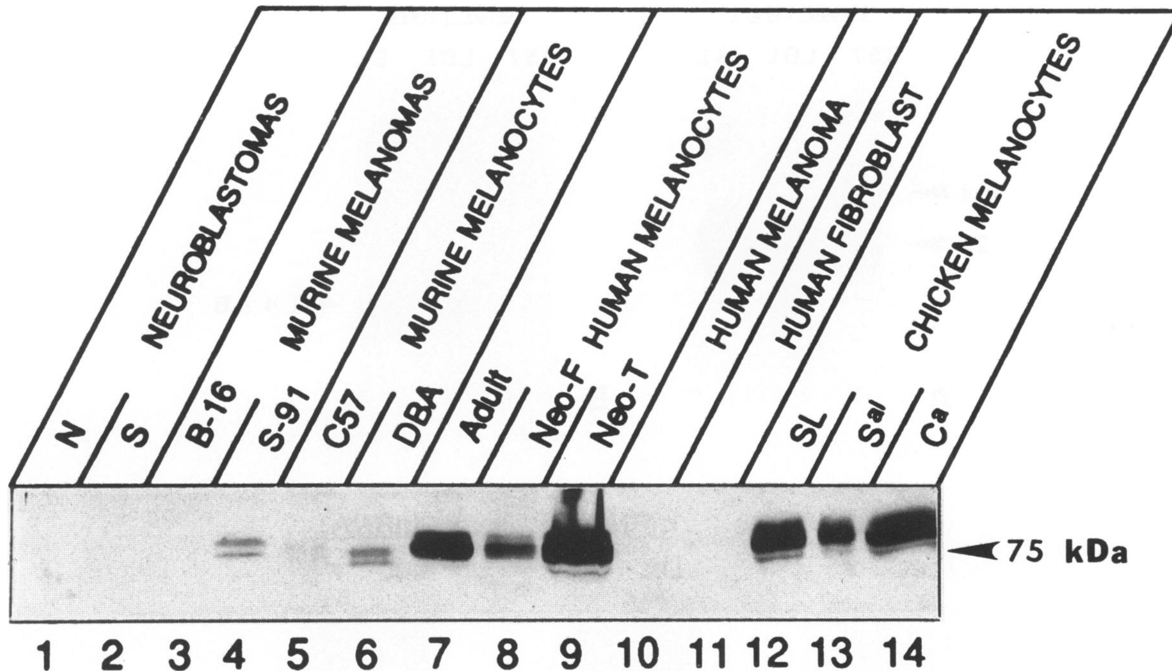

Figure 6. *SL autoantibodies recognize 65- to 80-kd melanocyte proteins from humans and murine melanocytes. Immunoblot of denatured lysates of mouse, human, and chicken cells (approximately 15 μg/lane) probed with SL autoantibodies (1:2000).* Lane 1, *N-type neuroblastoma;* lane 2, *S-type neuroblastoma;* lane 3, *B16 murine melanoma (originally isolated from C57BL/6J);* lane 4, *Cloudman S91 murine melanoma (originally isolated from F1 (DBA × C);* lane 5, *C57BL/6J mouse melanocytes;* lane 6, *DBA mouse melanocytes;* lane 7, *human melanocytes derived from adult tissue (cultured with leukotriene* C_4*),* lane 8, *human melanocytes derived from neonatal tissue (cultured with human recombinant basic fibroblast growth factor);* lane 9, *human melanocytes derived from neonatal tissue (cultured with phorbol 12-myristate 13-acetate);* lane 10, *human melanoma derived from adult amelanotic tissue that is negative for a TRP-1 transcript on Northern blots;* lane 11, *human fibroblasts (mouse fibroblasts and chicken fibroblasts were also negative, results not shown);* lane 12, *SL chicken melanocytes;* lane 13, *TYR-positive albino* (S^{al}) *chicken melanocytes; and* lane 14, *TYR-negative albino* (c^{a}) *chicken melanocytes. All cells and sources are described in Materials and Methods.*

SL autoantibodies did not recognize proteins from an amelanotic human melanoma that is negative for a TRP-1 RNA transcript and protein and positive for TYR, TRP-2, G3PDH transcripts (results not shown). The lysates from fibroblasts of all three species and the lightly pigmented murine B16 melanoma cells were negative for SL autoantibody binding. This experiment supports the hypothesis that the primary SL antigen is TRP-1.

Specific Transcripts for TRP-1 and TRP-2 Can Be Identified on Northern Blots of Chicken Melanocyte RNA

As this is the first report that suggests that chicken melanocytes express TRP-1 and TRP-2 homologues, we tested for the presence of potential chicken TRP-1 and TRP-2 RNA transcripts (see Table 2 for a description of the probes). The mouse TRP-1 probe recognizes a 2.1-kb band on the lanes of total chicken melanocyte RNA and the expected larger band of 3.1 kb on the mouse RNA (Figure 7A). The mouse TRP-1 band at 3.1 kb is faint and believed to be the result of lower loading levels of mouse RNA, as confirmed by reprobing the blots with the human G3PDH fragment, which shows that the relative level of RNA was much lower for the mouse than the chickens (Figure 7B). As the 2.1-kb band was in the vicinity of the 18S ribosomal RNA, mRNA was collected from wild-type melanocytes and chicken fibroblasts and probed with mammalian TRP-1 cDNA to rule out cross-hybridization to chicken 18S ribosomal RNA. Both the mouse (results not shown) and the human TRP-1 probes identified a 2.1-kb band (Figure 7C). These mRNA blots were reprobed with the mouse TRP-2 probe, which recognizes a chicken melanocyte transcript of 3.4 kb (Figure 7D). The chicken mRNA was also probed with a human TYR probe. This probe recognized a chicken transcript of 2.4 kb (Figure 7E) that was approximately the same size as the 2.5-kb chicken TYR transcript reported by Mochii et al.[27]

Discussion

TRP-1 was first described and studied as the mouse *brown* (or *b*-locus) protein and has historically been hypothesized to affect several melanogenic functions, eg, TYR activity and melanosomal shape and size, as well as to provide a structural framework for the attachment of TYR to the melanosomal matrix (re-

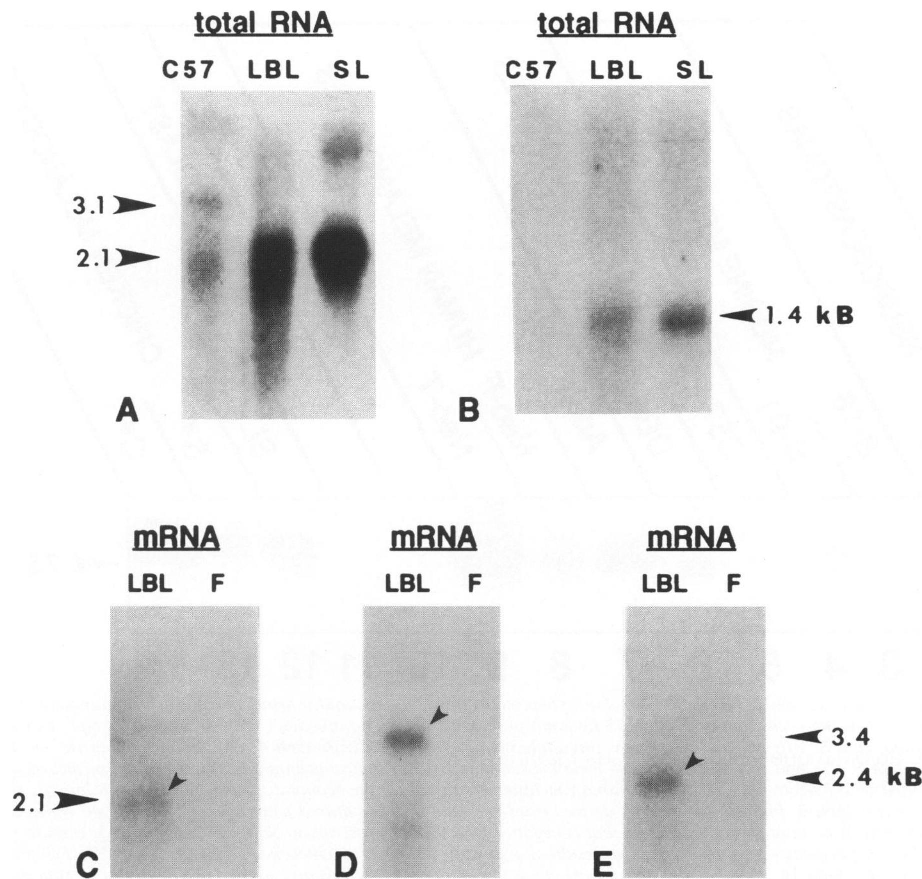

Figure 7. *Specific candidates for TRP-1 and TRP-2 can be identified on Northern blots of chicken melanocyte RNA when probed with cDNA fragments from mouse and human TRP-1 and mouse TRP-2. Total RNA and/or mRNA was isolated from cultured chicken melanocytes (LBL, Light Brown Leghorn), cultured chicken fibroblasts, and mouse melanocytes (C57), separated on an agarose-formaldehyde gel, blotted onto nylon membranes, and probed with ^{32}P-labeled TRP cDNA and human G3PDH cDNA.* A: *Total RNA from chicken melanocytes (approximately 10 μg) and from C57 mouse melanocytes (approximately 1 μg) probed with mouse TRP-1.* B: *The blot from* (A) *was stripped and reprobed with a human G3PDH cDNA.* C: *mRNA from wild-type chicken melanocytes (1 μg) and SL fibroblasts (0.6 μg) probed with human TRP-1.* D: *The blot from* (C) *was stripped and reprobed with mouse TRP-2.* E: *The blot from* (D) *was stripped and reprobed with human TYR.*

viewed by Silvers[28]). The molecular sequence of this protein was first determined by Shibahara et al[11] and considered a candidate cDNA sequence for TYR. Subsequently, this cDNA was shown to map near the murine *b*-locus in mice and the protein renamed TRP-1 because of its close homology with TYR.[29] Mutations at the *brown* locus of mice result in a brown coat color compared with wild-type coats, which are black.[28,30] It has been shown that a change from brown to black pigmentation develops when wild-type TRP-1 cDNA is transfected into light brown (melanin-b) cells.[31] Several enzymatic activities have recently been proposed for TRP-1, including tyrosine hydroxylase activity[32,33] and dihydroxyindol-2-carboxylic acid oxidase activity.[34] Recent studies also indicate that TRP-1 may affect or regulate the tyrosine hydroxylase activity of tyrosinase.[33,35]

In this study, we have shown that the SL autoantibodies from the chickens we tested do not appear to recognize chicken TYR or chicken/mammalian TRP-2 (DOPAchrome tautomerase). We have also shown that the primary SL antigen in mammalian melanocytes is TRP-1. These conclusions were reached by the following observations. First, SL autoantibodies recognize proteins of molecular mass different from the candidate chicken TYR and TRP-2. Second, the SL antigen exhibited the same pattern of post-translational processing as TRP-1 in human melanocytes. In addition, the SL autoantigen in chicken melanocytes showed a similar rate of synthesis and number of isoforms to TRP-1. And third, human melanocyte lysates depleted of TRP-1 also lost SL autoantibody immunoreactivity. We also observed that immunoblots of a human melanoma, negative for a TRP-1 transcript, were also negative for SL autoantibody reactivity. Northern blots probed with mammalian cDNA fragments of TRP-1 and TRP-2 show that cultured chicken melanocytes express candidate

transcripts for chicken TRP-1 and TRP-2 homologues. This is the first report that suggests and demonstrates that chicken melanocytes express TRP-1 and TRP-2. These results indicate that the SL chicken melanocyte autoantigen may be a chicken homologue of human gp75 and the mouse *b*-locus protein.

Additional evidence that the SL autoantibodies do recognize mammalian TRP-1 was found with melanocytes cultured from a human TYR-positive albino patient exhibiting the Brown phenotypic subtype that has been previously shown to completely lack TRP-1 protein and TRP-1 message.[36] These melanocytes do express both TYR and TRP-2 (message and protein).[36] Immunoblots of cell lysates from these TRP-1-deficient albino melanocytes completely lacked SL autoantibody reactivity, whereas immunoblots of melanocyte lysates from an unaffected twin (which has mRNA for TRP-1) had equivalent bands with both PEP2 and SL autoantibodies (Boissy et al, manuscript in preparation).

It was surprising to us when we found that the SL autoantibodies appear to recognize only mammalian TRP-1. One of the primary hypotheses for the pathogenesis of SL autoimmune vitiligo proposes that the melanocytes are eliminated either by self-destruction or destruction by the immune system (reviewed by Smyth[2]). When we found SL autoantibodies in SL chicks, which remained in a partial state of pigmentation for several weeks, we hypothesized that these autoantibodies were more likely the result of mass destruction of melanocytes[8] than a pathogenic component. In this scenario, the release of large quantities of previously sequestered antigens would prime the immune system to produce melanocyte-specific antibodies in every chicken with a vitiliginous condition. However, this would lead us to believe that we should find the SL autoantibodies recognizing other melanocyte-specific molecules in addition to TRP-1. Because of the limited sampling size for these types of experiments, we cannot completely rule out the possibility that some vitiliginous chickens could produce SL autoantibodies that would recognize additional melanocyte proteins. We have not yet specifically tested for antibodies of low titer or low affinity to other melanocyte proteins or tested for increased levels of autoantibodies for housekeeping proteins in vitiliginous chickens. On the basis of the experiments presented here and results from previously reported experiments,[8] we have determined that the autoantibodies that recognize TRP-1 are the predominant type. These results are consistent with the observations made by other investigators including Halaban and Moellmann,[37] who discuss the observation that TRP-1 is a highly immunogenic protein in both humans and mammals. These authors point out that, when melanocyte cell lysates or even purified TYR proteins are used as immunogens, the resulting antibodies are primarily directed against TRP-1.

Interestingly, the human homologue of the mouse *b* protein, gp75, was also originally identified with serum autoantibodies.[38,39] The serum of a melanoma patient was screened for autoantibodies that recognized melanoma and melanocyte antigens.[38] These autoantibodies primarily recognized a 70- to 75-kd melanocyte protein that was termed gp75. Subsequently, gp75 was identified as the human homologue of the mouse *b* (*brown*) protein.[39]

The serum from certain human vitiliginous patients have been shown to contain cytotoxic autoantibodies as detected by complement-mediated cytotoxicity assays and antibody-dependent cytotoxic cell-mediated assays.[40] Autoantibodies from this type of serum have also been shown to bind to cell surface molecules of pigment cells as determined by enzyme-linked immunosorbent assays with intact pigment cells.[41] Numerous pigment cell antigens have been detected with autoantibodies from human vitiliginous patients.[42,43] These autoantibodies are primarily directed against pigment cell antigens of 35, 40 to 45, 75, 85, 90, 150,[42] and 240 to 250 kd reported by Naughton et al.[43] Song et al[44] have recently demonstrated that the 75-kd melanocyte-specific antigen, against which a significant number of vitiligo patients express an autoantibody, is TYR. Whether TRP-1 can also be an autoantigen in human vitiligo needs to be further explored.

Recently, a report by Giacomini et al[45] has suggested that TRP-1 may be present, at least transiently, on the plasma membrane of human melanoma cells. Searle et al[46] showed that vitiliginous SL autoantibodies do recognize proteins on the surface of chicken melanocytes. If TRP-1 is an integral plasma membrane component of chicken melanocytes, it could directly play a specific role in mediating melanocyte destruction by humoral immunological mechanisms. However, we have not determined at this time whether the SL autoantibodies that recognize TRP-1 are the same as the autoantibodies that recognize proteins on the cell surface. The determination of the cytotoxic potential of the anti-TRP-1 SL autoantibodies combined with the verification of the plasma membrane localization of TRP-1 will be important in the determination of the melanocyte contribution to its own immunodestruction. If TRP-1 is located on the plasma membrane, it will be interesting to determine the role

cell surface expression might play in normal and in abnormal pigmentation.

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References

1. Ortonne J-P, Bose SK: Vitiligo: Where do we stand? Pigment Cell Res 1993, 6:61–72
2. Smyth JR Jr: The Smyth chicken: A model for autoimmune amelanosis. CRC Crit Rev Poultry Biol 1989, 2:1–19
3. Boissy RE, Lamoreux ML: Animal models of an acquired pigmentary disorder: vitiligo. Proc XIII Int Pigment Cell Conf, Tucson, AZ, 1986. Edited by J Bagnara. New York, Alan R. Liss, 1988, pp 207–218
4. Boissy RE, Lamont SJ, Smyth JR Jr: Persistence of abnormal melanocytes in immunosuppressed chickens of the autoimmune "DAM" line. Cell Tissue Res 1984, 235:663–668
5. Lamont SJ, Smyth JR Jr: Effect of busectomy on development of a spontaneous postnatal amelanosis. Clin Immunol Immunopathol 1981, 21:407–411
6. Boyle ML III, Pardue SL, Smyth JR Jr: Effect of corticosterone on the incidence of amelanosis in Smyth delayed amelanotic line chickens. Poultry Sci 1987, 66:363–367
7. Pardue SL, Fite KV, Bengston L, Lamont SJ, Boyle SJ, Boyle ML III, Smyth JR Jr: Enhanced integumental and ocular amelanosis following the termination of cyclosporin administration. J Invest Dermatol 1988, 88:758–761
8. Austin LM, Boissy RE, Jacobson BS, Smyth JR Jr: The detection of melanocyte autoantibodies in the Smyth chicken model for vitiligo. Clin Immunol Immunopathol 1992, 64:112–120
9. Hearing VJ, Tsukamoto K, Urabe K, Kameyama K, Montague PM, Jackson IJ: Functional properties of cloned melanogenic proteins. Pigment Cell Res 1992, 5:264–270
10. Halaban R, Pomerantz SH, Marshall S, Lambert DT, Lerner AB: Regulation of tyrosinase in human melanocytes grown in culture. J Cell Biol 1983, 97:480–488
11. Shibahara S, Tomita Y, Sakakura T, Nager C, Chaudhuri B, Muller R: Cloning and expression of cDNA encoding mouse tyrosinase. Nucleic Acids Res 1986, 14:2413–2427
12. Jackson IJ, Chambers DM, Tsukamoto K, Copeland NG, Gilbert DJ, Jenkins NA, Hearing VJ: A second tyrosinase-related protein, TRP2, maps to and is mutated at the mouse slaty locus. EMBO J 1992, 11:527–535
13. Yamamoto H, Brumbaugh JA: Purification and isoelectric heterogeneity of chicken tyrosinase. Biochim Biophys Acta 1984, 800:282–290
14. Oetting W, Langner K, Brumbaugh JA: Detection of melanogenic proteins in cultured chick-embryo melanocytes. Differentiation 1985, 30:40–46
15. Boissy RE, Halaban R: Establishment of proliferative pure cultures of pigmented chicken melanocytes from neural tubes. J Invest Dermatol 1985, 84:158–161
16. Tamura A, Halaban R, Moellmann G, Cowan JM, Lerner MR, Lerner AB: Normal murine melanocytes in culture. In Vitro Cell Dev Biol 1987, 23:519–522
17. Swope VB, Abdel-Malek ZA, Kassem L, Nordlund JJ: Interleukins 1α and 6 and tumor necrosis factor-α are paracrine inhibitors of human melanocyte proliferation and melanogenesis. J Invest Dermatol 1991, 96:180–185
18. Medrano EE, Nordlund JJ: Successful culture of adult human melanocytes from normal and vitiligo donors. J Invest Dermatol 1990, 95:441–445
19. Guerra L, Mordoh J, Slavutsky I, Larripa I, Medrano EE: Characterization of IIB-MEL-J: A new and highly heterogeneous human melanoma cell line. Pigment Cell Res 1989, 2:504–509
20. Abdel-Malek ZA, Kreutzfeld KL, Hadley ME, Bregman MD, Hruby VJ, Meyskens FL Jr: Long term and residual melanotropin-stimulated tyrosinase activity in S91 melanoma cells is density dependent. In Vitro Cell Dev Biol 1986, 22:75–81
21. Foley J, Cohn SL, Salwen HR, Chagnovich D, Cowan J, Mason KL, Parysek LM: Differential expression of N-*myc* in phenotypically distinct subclones of a human neuroblastoma cell line. Cancer Res 1991, 51:6338–6345
22. Jiménez M, Maloy WL, Hearing VJ: Specific identification of an authentic clone for mammalian tyrosinase. J Biol Chem 1989, 264:3397–3403
23. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate, phenol-chloroform extraction. Anal Biochem 1987, 162:156–159
24. Maniatis T, Fritsch EF, Sambrook J: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press, 1987
25. Tsukamoto K, Jackson IJ, Urabe K, Montague P, Hearing VJ: A second tyrosinase related protein, TRP-2, is a melanogenic enzyme termed dopa-chrome tautomerase. EMBO J 1992, 11:519–526
26. Vijayasaradhi S, Doskoch PM, Houghton AN: Biosynthesis and intracellular movement of the melanosomal membrane glycoprotein gp75, the human *b* (*Brown*) locus product. Exp Cell Res 1991, 196:233–240
27. Mochii M, Iio A, Yamamoto H, Takeuchi T, Eguchi G:

Isolation and characterization of a chicken tyrosinase cDNA. Pigment Cell Res 1992, 5:162–167
28. Silvers WK: The Coat Colors of Mice. A Model for Mammalian Gene Action and Interaction. New York, Springer-Verlag, 1979
29. Jackson IJ: A cDNa encoding tyrosinase-related protein maps to the *brown* locus in mice. Proc Natl Acad Sci USA 1988, 85:4392–4396
30. Zdarsky E, Favor J, Jackson IJ: The molecular basis of *brown*, an old mouse mutation, and of an induced revertant to wild type. Genetics 1990, 126:443–449
31. Bennett DC, Huszar D, Laipis PJ, Jaenisch R, Jackson IJ: Phenotypic rescue of mutant *brown* melanocytes by a retrovirus carrying a wild-type tyrosinase-related protein gene. Development 1990, 110:471–475
32. Jiménez M, Tsukamoto K, Hearing VJ: Tyrosinases from two different loci are expressed by normal and by transformed melanocytes. J Biol Chem 1991, 266: 1147–1156
33. Zhao H, Zhao Y, Nordlund JJ, Boissy RE: Human TRP-1 has tyrosine hydroxylase activity but no dopa oxidase activity. Pigment Cell Res 1994, 7:131–140
34. Jiménez-Cervantes C, Solano F, Kobayashi T, Urabe K, Hearing VJ, Lozano JA, García-Borrón JC: A new enzymatic function in the melanogenic pathway: the DHICA oxidase activity of tyrosinase related protein-1 (TRP1). J Biol Chem 1994, 269:17993–18001
35. Kameyama K, Takemura T, Hamada Y, Sakai C, Kondoh S, Nishiyama S, Urabe K, Hearing VJ: Pigment production in murine melanoma cells is regulated by tyrosinase, tyrosinase-related protein-1 (TRP-1), DOPAchrome tautomerase (TRP-2), and a melanogenic inhibitor. J Invest Dermatol 1993, 100:126–131
36. Boissy RE, Zhao H, Boissy YL, Austin LM, King RA, Nordlund JJ: Melanocytes from a *brown* oculocutaneous albino lack expression of TRP-1 and exhibit reduced insoluble melanin and aberrant tyrosine hydroxylase activity. Pigment Cell Res 1993, 6:275–276 (Abstr)
37. Halaban R, Moellmann G: Recent advances in the molecular biology of pigmentation: mouse models. Pigment Cell Res 1992, 2(Suppl):67–78
38. Mattes J, Thomson TM, Old LJ, Lloyd KO: Pigmentation-associated, differentiation antigen of human melanoma defined by a precipitating antibody in human serum. Int J Cancer Res 1983, 32:717–721
39. Vijayasaradhi S, Bouchard B, Houghton AN: The melanoma antigen gp75 is the human homologue of the mouse *b* (*Brown*) locus gene product. J Exp Med 1990, 171:1375–1380
40. Norris DA, Kissinger GM, Naughton GM, Bystryn J-C: Evidence for immunologic mechanisms in human vitiligo: patients' sera induce damage to human melanocytes *in vitro* by complement-mediated damage and antibody-dependent cellular cytotoxicity. J Invest Dermatol 1988, 90:783–789
41. Harning R, Cui J, Bystryn JC: Relationship between the incidence and level of pigment cell antibodies and disease activity in vitiligo. J Invest Dermatol 1991, 97: 1078–1080
42. Cui J, Harning R, Henn M, Bystryn J-C: Identification of pigment cell antigen defined by vitiligo antibodies. J Invest Dermatol 1992, 98:162–165
43. Naughton GK, Eisinger M, Bystryn J-C: Antibodies to normal human melanocytes in vitiligo. J Exp Med 1983, 158:246–251
44. Song YH, Connor E, Li Y, Zorovich B, Balducci P, Maclaren N: The role of tyrosinase in autoimmune vitiligo. Lancet 1994, 344:1049–1052
45. Giacomini P, Fraioli R, Cuomo M, Natali PG: Membrane compartmentalization of melanosomal gp75. J Invest Dermatol 1992, 98:340–342
46. Searle EA, Austin LM, Boissy YL, Zhao H, Nordlund JJ, Boissy RE: Smyth chicken melanocyte autoantibodies: cross-species recognition, *in vivo* binding, and plasma membrane reactivity of the antiserum. Pigment Cell Res 1993, 6:145–157
47. Fuller BB, Lunsford JB, Iman DS: Melanocyte-stimulating hormone regulation of tyrosinase in Cloudman S-91 mouse melanoma cell cultures. J Biol Chem 1987, 262:4024–4033
48. Tomita Y, Shibahara S, Takeda A, Okinaga S, Matsunaga J, Tagami H: The monoclonal antibodies TMH-1 and TMH-2 specifically bind to a protein encoded at the murine *b*-locus, not to the authentic tyrosinase encoded at the *c*-locus. J Invest Dermatol 1991, 96:500–504
49. Bouchard B, Fuller BB, Vijayasaradhi S, Houghton AN: Induction of pigmentation in mouse fibroblasts by expression of human tyrosinase cDNA. J Exp Med 1989, 169:2029–2042