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Identification and Cloning of Genes Associated with the Guinea Pig Skin Delayed–Type Hypersensitivity Reaction[1]

De Yang,[2] Kumiko Nakada-Tsukui, Masashi Ohtani, Riko Goto, Teizo Yoshimura,[2] Yoshiro Kobayashi, and Naoko Watanabe[3]

Department of Biomolecular Science, Faculty of Science, Toho University, 2-2-1 Miyama, Funabashi, Chiba 274-8510

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Although the cellular and molecular mechanisms underlying the delayed-type hypersensitivity (DTH) reaction have been investigated, the functions of infiltrating leukocytes and skin resident cells in the elicitation phase of the DTH reaction are not completely understood. To gain more insight into the role of these cells in the DTH reaction, we identified about 250 cDNA fragments showing elevated expression during the DNCB-induced guinea pig skin DTH reaction by differential display analysis. Characterization of 50 of them led to the identification of 28 genes whose expression was elevated in the DNCB-induced DTH reactive tissue. Sequencing of the 28 cDNA fragments and homology search analysis demonstrated that 10 of them represented known genes, some of which, in particular elafin (an elastase inhibitor) and ferritin, are considered to play roles in the DTH reaction. The other 18 fragments are probably derived from unknown genes. Cloning of the cDNAs of one of these genes indicated that it is that for guinea pig tryptophanyl-tRNA synthetase (WRS), a protein found to be induced by interferon-γ and upregulated during the late stages of mononuclear phagocyte maturation *in vitro*. Strong induction of the WRS gene during the DTH reaction suggests its involvement in the *in vivo* immune response.

Key words: delayed-type hypersensitivity, differential display analysis, guinea pig, skin, tryptophanyl-tRNA synthetase.

Delayed-type hypersensitivity (DTH), an important *in vivo* manifestation of the cell-mediated immune response, is characterized by predominant infiltration of leukocytes including antigen-specific T cells and antigen-nonspecific effector cells, fibrin deposition, as well as augmentation of vascular permeability at the site of antigen application (*1*, *2*). Traditional DTH reactions include the tuberculin skin reaction and hapten-induced contact hypersensitivity. In both cases, Langerhans cells, which are the principal antigen-presenting cells in the skin, are crucial for development of the DTH reaction (*3*, *4*). After the first application of an antigen (sensitization), activated Langerhans cells migrate to local lymph nodes, where they present the antigen to and stimulate the clonal expansion of specific T lymphocytes (*3*–*5*). A second application of the same antigen (elicitation) rapidly causes the infiltration and activation of antigen-specific memory T cells and antigen-nonspecific lymphocytes, which, in their turn, amplify the inflammatory reaction through the release of a variety of potent mediators with chemotactic and activating effects on other inflammatory cells (*3*, *4*, *6*). The products of activated infiltrating leukocytes, resident cells, and peripheral nerve endings at the site of elicitation are considered to function together to produce the DTH reaction.

Molecules including cytokines, chemokines, adhesion molecules, and others have been shown to be involved in the sensitization, elicitation, and modulation of the DTH reaction. Treatment *in vivo* with anti–ICAM-1, anti–LFA-1 (*7*, *8*), anti–IL-8 (*9*), anti–IL-12 (*10*), IL-1 receptor antagonist (*11*), or IL-10 (*12*), and knock-out of both E- and L-selectins (*13*), CD4 (*14*), or interferon (IFN)-γ receptor (*15*) were shown to suppress the DTH reaction, showing that ICAM-1, LFA-1, IL-8, IL-12, IL-1, E-selectin, L-selectin, CD4, and IFN-γ are indispensable or essential for DTH induction, while IL-10 and IL-1 receptor antagonists act to downmodulate the DTH reaction. The capacity of en-dogenous IL-10 to inhibit the skin DTH reaction is further supported by the fact that compared with wild type mice, mice with targeted disruption of the IL-10 gene exhibited an exaggerated DTH reaction in both magnitude and duration (*16*). IL-1 was the first cytokine to be induced in the skin by an allergen (*17*). Other cytokines as well as chemokines such as IL-6 (*12*), GM-CSF (*18*), IL-5 (*19*), MIP-1α (*20*), G-CSF (*21*), IP-10 (*22*), MCP-1 (*22*), IL-2 (*8*, *12*, *14*, *19*), and TNFα (*12*, *14*, *19*) have also been reported to be involved in development of the DTH reaction. We recently demonstrated that intradermal injection of neutralizing antibodies against TNFα or macrophage chemotactic factor partially suppressed elicitation of the guinea pig skin DTH

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[2] Present address: Frederick Cancer Research and Development Center, National Cancer Institute, National Institutes of Health, Frederick, MD21702-1201, USA.

[3] To whom correspondence should be addressed. Tel: +81-47-472-7696, Fax: +81-47-472-7696, E-mail: naokow@biomol.sci.toho-u.ac.jp

Abbreviations: DTH, delayed-type hypersensitivity; DNCB, dinitrochlorobenzene; SIC, skin-infiltrating cells; SRC, skin-resident cells; Con A, concanavalin A; TGC, thioglycollate medium; LPS, lipopolysaccharide; WRS, tryptophanyl-tRNA synthetase.

reaction (*23, 24*), providing additional evidence that these cytokines are involved in development of the DTH reaction.

One of the obvious characteristics of the DTH reaction is leukocyte infiltration (*1, 2*). We observed that about 40% of the leukocytes infiltrating into the DTH reaction site in guinea pigs were of the monocyte/macrophage lineage (*25*). In a kinetic study involving a gelatin sponge mouse model, it was shown that neutrophils were the first leukocytes to appear at the DTH-reactive site, followed by an increase in lymphocytes and then monocytes (*19*). Although the cellular and molecular mechanisms underlying the DTH reaction have been intensively investigated, the functions of infiltrating leukocytes and skin resident cells in the elicitation phase of the DTH reaction are not completely understood. To gain more insight into the role of these cells in the DTH reaction, we tried to identify, by means of the differential display technique (*26*), the genes whose expression is elevated during elicitation of the skin DTH reaction in the guinea pig. We chose the guinea pig as a model for a DTH reaction, mainly because this model is considered the best "predictive" rodent model for studying the DTH reaction (*27*).

EXPERIMENTAL PROCEDURES

Induction of the DTH Reaction—Female Hartley albino guinea pigs, purchased from SLC (Shizuoka), were housed under conventional clean conditions at the Animal Research Center of Toho University, Narashino Campus, for at least one week before use to confirm the absence of disease. The guinea pigs were used at 6–7 weeks of age.

For induction of the DTH reaction, guinea pigs were sensitized by s.c. injection, into their four foot pads (0.4 ml/head), of dinitrochlorobenzene (DNCB, 100 μg) emulsified with Freund complete adjuvant (FCA) in the ratio of 1:1. Two weeks later, 2.4 ml of a DNCB solution (in ethanol) or ethanol (as a control) alone was painted onto an area of shaved flank skin on the sensitized guinea pigs. At various times afterwards, guinea pigs were sacrificed and the flank skin was immediately excised for experiments.

Isolation of RNA from Skin-Infiltrating and Skin-Resident Cells—The preparation of skin-infiltrating cells (SIC) and skin-resident cells (SRC) was performed in almost the same way as previously described (*25*). Briefly, excised control or DTH-reactive skin was trimmed free of any subcutaneous tissue (including soft connective tissue and vasculature), cut into 1 mm cubes in Hanks solution, and subsequently subjected to digestion for 15 min at 37°C with the combination of collagenase (Nitta Gelatin, Osaka) and DNase I (Sigma, USA) at final concentrations of 4 mg/ml and 1 μg/ml, respectively. The digested mixture was then passed through 150-mesh filters, followed by centrifugation at 1,200 rpm for 7 min to pellet the cells. Erythrocytes in the cell pellet were removed by treatment with Tris-buffered NH_4Cl (0.83%). After 3 washes with Hanks solution, the cells were suspended in phosphate-buffered saline and then their number was determined with a hemocytometer. SIC contained macrophages, neutrophils, lymphocyte-like cells, and large and flattened cells, whereas SRC contained a much larger percentage of large and flattened cells, in good agreement with our previous results (*25*). The viability of the isolated SIC or SRC was usually above 90%, as determined by means of trypan blue exclusion. Crude RNAs were isolated from SIC and SRC by use of QIAshredders (QIAGEN, Germany) according to the manufacturer's instructions. Then 50 μg portions of the crude RNA samples were incubated with 10 units of RNase-free DNase I (Boehringer, Germany) in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, and 1.5 mM $MgCl_2$ in the presence of 10 units of placental ribonuclease inhibitor (Toyobo, Osaka) for 30 min at 37°C, followed by recovery with an RNeasy total RNA kit (QIAGEN). The purified SIC- and SRC-RNAs were used later in differential display experiments. For the extraction of total RNA from skin tissue, excised skin was trimmed free of any subcutaneous tissue (including soft connective tissue and vasculature), minced into 1 mm cubes in a 4 M guanidinium thiocyanate solution, and then homogenized with a Polytron (Kinematica, Switzerland). The total RNA in the homogenate was then extracted according to the method of Chomczynski and Sacchi (*28*), and used for Northern blot hybridization.

Differential Display Analysis—(a) Primers. Arbitrary primers were designed by adding the GCGTGAATTC sequence (containing an *Eco*RI site) to 26 10-mer deoxyoligonucleotides, as reported (*29*). Anchored primers were designed by adding the GCGCAAGCTT sequence (containing a *Hin*dIII site) to three kinds of one-base anchored 10 mer oligo dT, as reported (*30*). The sequences of all the arbitrary and anchored primers used in this study are summarized in Table I. (b) Reverse transcription. A microfuge tube, containing 25 μl of diethylpyrocarbonate-treated H_2O, 2 μl of human placental reverse transcriptase inhibitor (33 units/μl; Toyobo), 3 μl of purified SIC- or SRC-RNA (0.2 mg/ml), 6 μl of a 0.2 mM dNTP mixture, 3 μl of 20 μM anchored primer (either HT11C, HT11G, or HT11A), and 12 μl of 5× reverse transcription buffer, was heated at 65°C for 5 min, and then incubated at 37°C for 10 min. After the addition of 9 μl of Moloney murine leukemia virus reverse transcriptase (20 units/μl, BRL, USA) to the tube, the mixture was further incubated at 37°C for 50 min. Finally, the reaction mixture was heated at 95°C for 5 min, and then used immediately or frozen at −70°C for later use. (c) PCR amplification and band separation. To each PCR tube, 3 μl of H_2O, 1 μl of a reverse transcription mixture, 1 μl of 10× Taq DNA polymerase buffer, 0.5 μl of 50 mM $MgCl_2$, 1 μl of a 40 μM dNTP mixture, 1 μl of 4 μM corresponding anchored primer, 1 μl of 4 μM arbitrary primer, 1 μl of Taq DNA polymerase (0.5 unit/μl; Boehringer), and 0.5 μl of 10 mCi/ml [α-^{35}S]dCTP (1,250 Ci/mmol; NEN, USA) were added, followed by overlaying with 30 μl of mineral oil. All solutions were kept on ice to avoid any nonspecific annealing and extension. The cycling parameters were as follows: 94°C for 1 min, 40°C for 4 min, and 72°C for 2 min for the first cycle, 94°C for 30 s, 60°C for 2 min, and 72°C for 1 min for another 35 cycles, followed by post-extension at 72°C for 5 min. The cycling reaction was performed with a PC-700 Program Temperature Control System (ASTEC, Fukuoka). The amplified cDNAs were then separated on a DNA sequencing gel (6% polyacrylamide, 8 M urea). At the end of the electrophoresis, the sequencing gel was blotted onto a piece of Whatman 3 MM paper, dried under vacuum, and then examined with a BAS1000 Bio-Imaging analyzer (Fuji Film, Tokyo). (d) Recovery and reamplification of target cDNA fragments. For comparison of the cDNA band patterns of SIC- and SRC-RNAs, the bands which only appeared for SIC-RNA were considered as target bands. The target bands were cut out from the dried gel. Each gel slice containing a target band was soaked in 100 μl of H_2O to allow rehydration. The cDNA was diffused out by boiling the tube for 15 min, followed by ethanol precipitation. A portion of the eluted cDNA was reamplified using the same primer set.

Determination of the Sequences of cDNA Fragments—The target cDNA fragments were inserted by A/T cloning into the pGEM-T vector (Promega, USA) or by direct cloning into the *Eco*RI- and *Hin*dIII-digested pGEM-3Z vector. DNA sequencing of cloned cDNA fragments with M13 universal or reverse primers was carried out with a Cy5™ AutoRead™ Sequencing kit (Amersham Pharmacia Biotech, Tokyo) and an automated ALFred DNA sequencer (Amersham Pharmacia Biotech).

The sequences of target cDNA fragments were compared with all the nonredundant sequence data recorded in the GenBank, EMBL, DDBJ, PIR, and SwissPort databases by means of the FASTA and BLAST computer programs, using a Supernig computer (National Institute of Genetics, Shizuoka).

Northern Blot Hybridization—The total RNA was separated on an agarose-formadehyde gel (1%) and then transferred to a piece of Hybond-C extra nitrocellulose membrane (Amersham Pharmacia Biotech). The filter was hybridized with a ^{32}P-labeled cDNA probe as described previously (*31*). The probes, cloned target cDNA fragments or a 1,800-bp guinea pig β-actin cDNA fragment cloned in our laboratory (data not shown), were labeled with 10 mCi/ml [α-^{32}P]dCTP (3,000 Ci/mmol; ICN, USA) by random priming using a *Bca*BEST™ Labeling kit (TaKaRa, Tokyo). The hybridized filter was processed with a BAS1000 Bio-Imaging analyzer (Fuji Film). Signal intensities were normalized as to that of β-actin mRNA on corresponding filters to correct for the RNA quantities loaded.

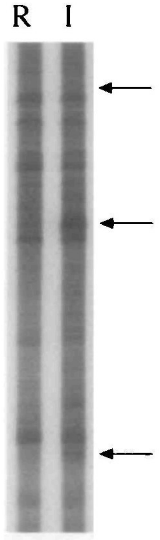

Fig. 1. **Parts of autoradiograms of differential display gels.** The anchored and arbitrary primers used were HT11C and EAP24 (Table I), respectively. The differentially expressed bands between SRC (R) and SIC (I) are indicated by arrows.

TABLE I. **Primer design for differential display.**[a]

No.	Sequence (5′ to 3′)	Abbreviation
Arbitrary primers		
1	GCGTGAATTCTACAACGAGG	(EAP1)
2	GCGTGAATTCTGGATTGGTC	(EAP2)
3	GCGTGAATTCCTTTCTACCC	(EAP3)
4	GCGTGAATTCTTTTGGCTCC	(EAP4)
5	GCGTGAATTCGGAACCAATC	(EAP5)
6	GCGTGAATTCAAACTCCGTC	(EAP6)
7	GCGTGAATTCTCGATACAGG	(EAP7)
8	GCGTGAATTCTGGTAAAGGG	(EAP8)
9	GCGTGAATTCTCGGTCATAG	(EAP9)
10	GCGTGAATTCGGTACTAAGG	(EAP10)
11	GCGTGAATTCTACCTAAGCG	(EAP11)
12	GCGTGAATTCCTGCTTGATG	(EAP12)
13	GCGTGAATTCGTTTTCGCAG	(EAP13)
14	GCGTGAATTCGATCAAGTCC	(EAP14)
15	GCGTGAATTCGATCCAGTAC	(EAP15)
16	GCGTGAATTCGATCACGTAC	(EAP16)
17	GCGTGAATTCGATCTGACAC	(EAP17)
18	GCGTGAATTCGATCTCAGAC	(EAP18)
19	GCGTGAATTCGATCATAGCC	(EAP19)
20	GCGTGAATTCGATCAATCGC	(EAP20)
21	GCGTGAATTCGATCTAACCG	(EAP21)
22	GCGTGAATTCGATCGCATTG	(EAP22)
23	GCGTGAATTCGATCTGACTG	(EAP23)
24	GCGTGAATTCGATCATGGTC	(EAP24)
25	GCGTGAATTCGATCATAGCG	(EAP25)
26	GCGTGAATTCGATCTAAGGC	(EAP26)
One-base anchored primers		
1	GCGCAAGCTTTTTTTTTTTC	(HT11C)
2	GCGCAAGCTTTTTTTTTTTG	(HT11G)
3	GCGCAAGCTTTTTTTTTTTA	(HT11A)

[a] The abbreviations used for the arbitrary and one-base anchored primers are: EAP, *Eco*RI-site attached arbitrary primer; HT11C (or G or A), *Hin*dIII-site attached oligo dT(11) deoxycytidylate (or deoxyguanylate or deoxyadenylate)-anchored primer.

TABLE II. **The identified genes differentially expressed during the DTH reaction.**

cDNA clone		Results of a homology search in nonredundant data banks using BLAST and FASTA
Name	Insert size (bp)	
f2-c1	167	unique
f2-c4	129	unique
f3-c1	141	elafin
f7-c1	124	unique, 90% homology with D31886*
f13-c1	149	mitochondrion cytochrome *b*
f31-c1	162	unique
f32-c1	201	unique
f32-c3	156	ribosomal RNA
f36-c1	90	MHC class II antigen
f36-c2	73	unique
f49-c1	515	ferritin heavy chain
f56-c1	253	unique, 70% homology with AA323500*
f56-c3	231	unique
f59-c1	358	unique, 65% homology with AA261572* & T67068*
f59-c2	159	*Hox*-1.7 protein
f59-c3	312	unique, 90% homology with AC0015*
f62-c1	194	unique
f62-c3	127	ribosomal L34 protein
f94-c1	184	unique, 60% homology with H11836*
f120-c1	246	unique, 80% homology with N53996*
f120-c2	275	unique
f127-c1	213	unique, 80% homology with AA211693*
f141-c1	160	unique
f141-c2	110	unique
f156-c2	124	unique
f211-c1	287	B cell activation gene
f232-c1	326	α chain of MHC class II antigen
f235-c2	170	T cell receptor

*Accession number of EST, STS, GSS, or HTGS sequence data.

Construction of a cDNA Library and cDNA Cloning—A guinea pig splenocyte–derived cDNA library was constructed by use of a lambda ZAPII predigested *Eco*RI/CIAP-treated vector kit with the GigapackIII gold packaging extract (Stratagene, USA). The cDNA was synthesized on the mRNA derived from guinea pig splenocytes stimulated with concanavalin A (Con A) for 6 h by use of a cDNA synthesis kit (Stratagene). For the cloning of specific cDNA, about 1 × 10^6 pfu was screened by routine plaque hybridization with a ^{32}P-labeled probe. The positive clones were converted into phagemids according to the *in vivo* excision procedure (Stratagene).

RESULTS

Differential Display Analysis—To identify genes in SIC which might be important in the guinea pig skin DTH reaction, gene expression at the mRNA level in SIC was compared with that in SRC by differential display analysis. When both SIC and SRC RNAs were reverse-transcribed using HT11C (Table I), and subsequently amplified with the combination of HT11C and EAP24 (Table I), three additional cDNA bands, each corresponding to the 3′ end of a mRNA species, were observed (Fig. 1), suggesting that three genes might be newly expressed in SIC during the DNCB-induced guinea pig skin DTH reaction. On differential display analysis in the same way, using combinations of the 3 anchored and 26 arbitrary primers (Table I), 250 bands were obtained.

Detection of Genes Expressed during the DNCB-Induced DTH Reaction—As it was impractical to simultaneously characterize all the 250 genes that were expressed in SIC within DTH-reactive skin tissue, 50 cDNA bands, amounting to 1/5 of those obtained, were randomly chosen for further characterization. In order to determine whether or not the 50 bands represented true differences between SIC and SRC, we performed Northern blot hybridization (*26*) using RNA isolated from 24-h sham-treated (control, left flank) and DNCB-elicited (right flank) skin tissue of the same guinea pigs, because it was very difficult to obtain sufficient amounts of SIC- and SRC-derived RNAs (data not shown). Using ^{32}P-labeled cDNA fragments amplified from the 50

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   1 GCGGACGAGCCCGACAGCCAGCTGCTTGCCTCCCCGCTGCAGCTATTCAACGGCATAGCG   60
      A  D  E  P  D  S  Q  L  L  A  S  P  L  Q  L  F  N  G  I  A
  61 GCCCAGGGGGAGCGCGTGCGGGCCCTCAAGGACGCAAAGGCGCCAAAGGATGACATCGAC  120
      A  Q  G  E  R  V  R  A  L  K  D  A  K  A  P  K  D  D  I  D
 121 TCTGCAGTCAAGTTGCTCTTGTCATTAAAAATGAACTACAAAGCCACCGTGGGGGAGGAT  180
      S  A  V  K  L  L  L  S  L  K  M  N  Y  K  A  T  V  G  E  D
 181 TACAACCCTGACTGCCCCCCGGGAACCCTGGCGCCTGGGACCAAGGGTGGCCAGGAGGAC  240
      Y  N  P  D  C  P  P  G  T  L  A  P  G  T  K  G  G  Q  E  D
 241 TGCGAGGACTTCGTGGACCCGTGGACAGTGCGGACGAGCAGCGCCAAAGGCATCGACTAT  300
      C  E  D  F  V  D  P  W  T  V  R  T  S  S  A  K  G  I  D  Y
 301 GACAAGCTTATAGTTCAGTTCGGGAGCAGTAAGATTGACAAAGAGCTGATCAACCGGATA  360
      D  K  L  I  V  Q  F  G  S  S  K  I  D  K  E  L  I  N  R  I
 361 GAGAGGGCCACCAAGCAGCGGCCACACCGCTTCCTGCGCAGAGGCGTCTTCTTCTCACAC  420
      E  R  A  T  K  Q  R  P  H  R  F  L  R  R  G  V  F  F  S  H
 421 AGAGATATGAACCAAGTGCTGGACGCCTATGAGAGCGGGAAGCCGTTTTACCTGTACACG  480
      R  D  M  N  Q  V  L  D  A  Y  E  S  G  K  P  F  Y  L  Y  T
 481 GGCCGGGGCCCCTCCTCCGAAGCCATGCACGTCGGCCACCTCATCCCGTTCATCTTTACC  540
      G  R  G  P  S  S  E  A  M  H  V  G  H  L  I  P  F  I  F  T
 541 AAGTGGCTGCAGGACGTGTTCAACGTGCCCCTGGTGGTCCAGATGTCCGACGACGAGAAG  600
      K  W  L  Q  D  V  F  N  V  P  L  V  V  Q  M  S  D  D  E  K
 601 TACCTGTGGAAGGACCTGACCCTGGAGCAGGCCTACGGCTACACCCTGGAGAACGCCAAG  660
      Y  L  W  K  D  L  T  L  E  Q  A  Y  G  Y  T  L  E  N  A  K
 661 GACATCATCGCCTGCGGCTTCGACATCAACAAGACCTTCATCTTCTCCGACCTGGAGTAC  720
      D  I  I  A  C  G  F  D  I  N  K  T  F  I  F  S  D  L  E  Y
 721 ATGGGGATGAGCCCAGGCTTCTACAAGAATGTGGTGAAGATTCAGAAGCACGTCACCTTC  780
      M  G  M  S  P  G  F  Y  K  N  V  V  K  I  Q  K  H  V  T  F
 781 AACCAGGTGAAGGGCATCTTCGGCTTCACCGACAGCGACTGCATCGGGAAGATCAGTTTC  840
      N  Q  V  K  G  I  F  G  F  T  D  S  D  C  I  G  K  I  S  F
 841 CCCGCCGTGCAGGCCGCGCCCTCCTTCAGCAACTCGTTCCCGCAGATCTTCCGGGACCGG  900
      P  A  V  Q  A  A  P  S  F  S  N  S  F  P  Q  I  F  R  D  R
 901 ACGGACATCCAGTGCCTCATCCCGTGTGCCATTGACCAGGATCCCTACTTCAGGATGACG  960
      T  D  I  Q  C  L  I  P  C  A  I  D  Q  D  P  Y  F  R  M  T
 961 CGGGACGTGGCCCCCAGGATCGGCTACCCGAAGCCAGCCCTGCTGCACTCCACCTTCTTC 1020
      R  D  V  A  P  R  I  G  Y  P  K  P  A  L  L  H  S  T  F  F
1021 CCCGCCCTGCAGGGCGCCCAGACCAAGATGAGCGCCAGCGACCCCAACTCCTCCATCTTC 1080
      P  A  L  Q  G  A  Q  T  K  M  S  A  S  D  P  N  S  S  I  F
1081 CTCACCGACTCGGCCAAGCAGATCAAGACCAAGGTCAATAAGCACGCGTTCTCCGGAGGC 1140
      L  T  D  S  A  K  Q  I  K  T  K  V  N  K  H  A  F  S  G  G
1141 CGGGACACCGTGGAGGAGCACCGGCAGTTCGGGGGCAACTGTGACGTGGACGTGTCCTTC 1200
      R  D  T  V  E  E  H  R  Q  F  G  G  N  C  D  V  D  V  S  F
1201 ATGTACCTGACCTTCTTCCTGGAGGACGATGACCGGCTGGAGCAGATCCGCAAGGACTAC 1260
      M  Y  L  T  F  F  L  E  D  D  D  R  L  E  Q  I  R  K  D  Y
1261 ACCAGCGGGGCCATGCTCACCGGAGAGCTCAAGAAGACCCTCATTGACGTCCTGCAGCCC 1320
      T  S  G  A  M  L  T  G  E  L  K  K  T  L  I  D  V  L  Q  P
1321 CTGATCGCCGAGCACCAGGCCCGGCGCAAGGAGGTCACCGACGAGATGGTGAAGGAGTTC 1380
      L  I  A  E  H  Q  A  R  R  K  E  V  T  D  E  M  V  K  E  F
1381 ATGACCCCCCGGCCCCTGTCCTTCCACTTCCAGTAGCGCCCCTCAGCGCCCCCGAGGCTG 1440
      M  T  P  R  P  L  S  F  H  F  Q  *
1441 CCGTCCGCGGTAATCCTAGGTCATTCCCGGCGCCTGCCAGCCCTGCATGTGTTACGGATT 1500
1501 CCGGTTCTTCCTCTGACGTCTGTCCTTCCTGTCACCTGGGTAATCGGGTACTGGCTCACG 1560
1561 TCGTGTGGCCAGATAGGAAGCCCACAGGAGGCTCCCCACATGGATCCCAGCCATGGCCTG 1620
1621 TGCCGCCCAGAGCCACCAGGATTTCAGGTGCACCCCCAAGATGCTCCACGAGAAACCACT 1680
1681 TCATTGTGTGTGTGTGGATGATCCAAACATCATCCTTCTAAAAGTACCATGGAAAATGGA 1740
1741 TGTGACAGTGTCATGGGCACAGGACATTGGAGCCCGTGCCCCTCAAAAAAGAAATTGACC 1800
1801 ACTCAGAGCTCTGCGGAGCACTTGACCAAGATTGGGTCTGGGGTATGGCTCCCTGCAGAG 1860
1861 CAGCTAGCCTAGATACGTGTCAGCAGGTGACAACCTAGTGCCCTGAGTAAGTAGTTGTCC 1920
1921 AGCATCTCAGCTGCCTGCACGCTGGCCCTGGTGTCACGGGTTTCCCGAGCTAGCCAGCTC 1980
1981 AAGACCAGGGGCGTCCTCACTCTGTGGATCATACTCCTCACCCCTAATGCGCAGGCTTTA 2040
2041 CAGCAAACAAGCCAGAACTCCCTCTGCCCAATCCGGGGAGCCCTTTACCCAGGAGACGCT 2100
2101 GAAACACTGTTACCTTGCAGATGCTTTTGACTCTTAACCTCAGCGGAAGTCAGCACAGAG 2160
2161 CCTTGGCAGGGCGTGCAGCTGGTGGACCTAACCGAACGCGCGTGGTGCCCAGGGCTCAG 2220
2221 CCTCCTGTGCGCAGTGACCTCTGATTCCATGTGTAGGCTCCATGGGCAGAGCCGCCCCTT 2280
2281 GGGGGAAAGTCCTGGGTGCCAAACTGACGGTCCTTGGACTTGCAGGTCCACACTGGGTGC 2340
2341 TCTGCCCTCCCGCACTCTCAGCCTAATAAACGAACTTTACTCCTGAAAAAAAA 2393
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Fig. 2. **Nucleotide sequence of guinea pig f120-c2 cDNA.** The 2,393-nt-long f120-c2 cDNA is presented, as is the 471 amino acid sequence deduced from an open reading frame. The *Eco*RI site-containing adapter sequences at both ends are omitted. The nt positions are numbered from the 5′-most end. The termination codon is denoted by an asterisk and the polyadenylation signal sequence is shaded. The nucleotide sequence corresponding to an insert of f120-c2 is underlined.

cDNA bands as probes, 28 fragments were confirmed to represent differentially expressed cDNAs in the 24-h DNCB-induced skin tissue as compared to the control skin tissue of the same guinea pigs.

We then cloned the 28 fragments into the pGEM vector, and determined their sequences. As shown in Table II, the 28 cloned fragments ranged from 73 bp to 515 bp after the removal of both arbitrary and anchored primer sequences at both ends. By means of both the FASTA and BLAST computer programs the sequences of the 28 cloned fragments were compared with all of the nonredundant DNA sequences previously reported in the GenBank, EMBL, DDBJ, PIR, and SwissPort databases. These results revealed that 18 cloned fragments were probably "unique," meaning that they did not exhibit significant homology with any reported DNA sequences. The other 10 cloned fragments were the counterparts of guinea pig genes, such as those for MHC antigen, T cell antigen receptor, elafin, mitochondrion cytochrome *b*, ferritin heavy chain, ribosomal RNA, ribosomal L34 protein, and Hox-1.7 protein.

Cloning of f120-c2 cDNA—We screened a cDNA library from guinea pig splenocytes with a ^{32}P-labeled insert fragment of f120-c2 (Table II), because mRNA for f120-c2 was highly induced during the DTH reaction. One of several positive clones, f120-c2-5, contained an insert of 2,393 bp (Fig. 2). In this clone there was only one possible open reading frame that could produce a peptide of 471 amino acids, a long 3′-untranslated region of 978 bp, and a polyadenylation signal sequence.

Comparison of the deduced amino acid sequence of f120-c2-5 (Fig. 2) with the PIR and SwissPort peptide databases revealed that an open reading frame exhibited 84% homology with human and mouse WRS (*32, 33*) (Fig. 3), apparently indicating that the cloned fragment from f120-c2 is the guinea pig WRS gene.

Expression of WRS mRNA—The kinetics of expression of WRS mRNA in the skin cells during the DTH reaction were examined (Fig. 4A). WRS mRNA was induced at 12 h after elicitation of the DNCB-induced guinea pig skin DTH reaction, and was still high at 24 and 48 h after elicitation. We next investigated the expression of WRS mRNA in the immune cells (Fig. 4B). WRS mRNA was expressed in spleen cells (lane 1) and thioglycollate medium (TGC)–elicited peritoneal macrophages (lane 4). When spleen cells were stimulated with Con A (lane 2) or lipopolysaccharide (LPS) (lane 3), or peritoneal macrophages with LPS (lane 5), the expression of WRS mRNA was upregulated. On the contrary, WRS mRNA was hardly detected in both unstimulated and Con A–stimulated thymic cells (lanes 6 and 7). WRS mRNA was also expressed in various tissues of guinea pig other than thymus, including spleen, brain, heart, lung, kidney, adrenal gland, uterus, ovary, bladder, and small intestine (data not shown). The level of WRS mRNA was not significantly different after elicitation (data not shown).

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Guinea pig    1:-ADEPDSQLLASPLQLFNGIAAQGERVRALKDAKAPKDDIDSAVKLLLSLKMNYKATVGE  59
Human         1:---MPNSEP-ASLLELFNSIATQGELVRSLKAGNASKDEIDSAVKMLVSLKMSYKAAAGE  56
Mouse         1:MADMPSGESCTSPLELFNSIATQGELVRSLKAGNAPKDEIDSAVKMLLSLKMSYKAAMGE  60
                    *      * * *** ** *** ** **   * ** ****** * **** ***  **

Guinea pig   60:DYNPDCPPG-TLAPGTKGGQ--EDCEDFVDPWTVRTSSAKGIDYDKLIVQFGSSKIDKEL 116
Human        57:DYKADCPPGNPAPTSNHGPDATEAEEDFVDPWTVQTSSAKGIDYDKLIVRFGSSKIDKEL 116
Mouse        61:EYKAGCPPGNPTAGRNCDSDATKASEDFVDPWTVRTSSAKGIDYDKLIVQPGSSKIDKEL 120
                 *   ****                ********* **************  *********

Guinea pig  117:INRIERATKQRPHRFLRRGVFFSHRDMNQVLDAYESGKPFYLYTGRGPSSEAMHVGHLIP 176
Human       117:INRIERATGQRPHHFLRRGIFFSHRDMNQVLDAYENKKPFYLYTGRGPSSEAMHVGHLIP 176
Mouse       121:INRIERATGQRPHRFLRRGIFFSHRDMNQILDAYENKKPFYLYTGRGPSSEAMHLGHLVP 180
                ******** **** ***** ********* *****  ***************** *** *

Guinea pig  177:FIFTKWLQDVFNVPLVVQMSDDEKYLWKDLTLEQAYGYTLENAKDIIACGFDINKTFIFS 236
Human       177:FIFTKWLQDVFNVPLVIQMTDDEKYLWKDLTLDQAYGDAVENAKDIIACGFDINKTFIFS 236
Mouse       181:FIFTKWLEDVFNVPLVIQMSDDEKYLWKDLTLEQAYSYTVENAKDIIACGFDINKTFIFS 240
                ******* ******** ** ************ ***    ********************

Guinea pig  237:DLEYMGMSPGFYKNVVKIQKHVTFNQVKGIFGFTDSDCIGKISFPAVQAAPSFSNSFPQI 296
Human       237:DLDYMGMSSGFYKNVVKIQKHVTFNQVKGIFGFTDSDCIGKISFPAIQAAPSFSNSFPQI 296
Mouse       241:DLEYMGQSPGFYRNVVKIQKHVTFNQVKGIFGFTDSDCIGKSSFPAVQAAPSFSNSFPKI 300
                ** *** * *** **************************** **** *********** *

Guinea pig  297:FRDRTDIQCLIPCAIDQDPYFRMTRDVAPRIGYPKPALLHSTFFPALQGAQTKMSASDPN 356
Human       297:FRDRTDIQCLIPCAIDQDPYFRMTRDVAPRIGYPKPALLHSTFFPALQGAQTKMSASDPN 356
Mouse       301:FRDRTDIQCLIPCAIDQDPYFRMTRDVAPRIGHPKPALLHSTFFPALQGAQTKMSASDPN 360
                ******************************** ***************************

Guinea pig  357:SSIFLTDSAKQIKTKVNKHAFSGGRDTVEEHRQFGGNCDVDVSFMYLTFFLEDDDRLEQI 416
Human       357:SSIFLTDTAKQIKTKVNKHAFSGGRDTIEEHRQFGGNCDVDVSFMYLTFFLEDDDKLEQI 416
Mouse       361:SSIFLTDTAKQIKSKVNKHAFSGGRDTVEEHRQFGGNCEVDVSFMYLTFFLEDDDRLEQI 420
                ******* ***** ************* ********** **************** ****

Guinea pig  417:RKDYTSGAMLTGELKKTLIDVLQPLIAEHQARRKEVTDEMVKEFMTPRPLSFHFQ 471
Human       417:RKDYTSGAMLTGELKKALIEVLQPLIAEHQARRKEVTDEIVKEFMTPRKLSFDFQ 471
Mouse       421:RKDYTSGAMLTGELKKTLIDVLQPLIAEHQARRKAVTEETVKEFMTPRQLSFHFQ 475
                **************** ** ************** ** * ******** *** **
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Fig. 3. **Amino acid sequence homology among guinea pig, human and mouse WRS.** The amino acid sequences of WRS from guinea pig f120-c2 cDNA (upper line, refer to Fig. 2), human (middle line) (*31*), and mouse (lower line) (*32*) are aligned.

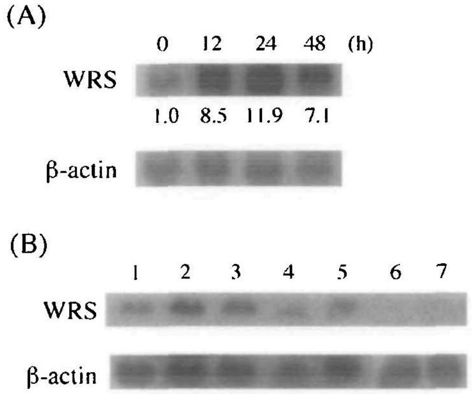

Fig. 4. **(A) Kinetic studies of WRS mRNA expression during the DNCB-induced guinea pig skin DTH reaction.** Guinea pigs sensitized 10 days previously were treated with DNCB for induction of the DTH reaction. At the times specified after the treatment, total RNA was extracted from guinea pig skin. Specific signals for WRS and β-actin were detected by Northern blot hybridization of the same filter. The time after induction and the level of WRS mRNA relative to the control (0 h) after normalization as to that of β-actin mRNA are shown at the top and middle, respectively. **(B) Expression of WRS mRNA in immune cells.** Total RNA was isolated from guinea pig cells and then Northern blot hybridization was performed. Lane 1, spleen cells; lane 2, spleen cells stimulated with 5 μg/ml Con A for 4 h; lane 3, spleen cells stimulated with 1 μg/ml LPS for 4 h; lane 4, TGC-elicited peritoneal macrophages; lane 5, TGC-elicited peritoneal macrophages stimulated with 1 μg/ml LPS for 4 h; lane 6, thymic cells; lane 7, thymic cells stimulated with 5 μg/ml Con A for 4 h.

DISCUSSION

DTH is characterized by the infiltration of a large number of leukocytes (*1–6*, *19*, *25*). Recent studies have established that many cytokines and chemokines (*3*, *9*, *11*, *12*, *15*, *24*, *34*), some adhesion molecules (*7*, *8*, *13*, *14*, *34*), and some neurohormones (*34*, *35*) participate in this process. However, it can be speculated that many other gene products are also involved. It is therefore considered that DTH must be a highly complicated process like many other *in vivo* body responses. To isolate the genes involved in the DTH reaction, differential display analysis was carried out to compare the gene expression profiles of SIC and SRC. We obtained 250 bands that seemed to be specifically expressed in SIC isolated from 24-h guinea pig DTH-reactive skin. Characterization of 50 randomly chosen cDNA bands revealed that 28 cDNA fragments probably represented genes which were expressed during the DNCB-induced guinea pig skin DTH reaction (Table II).

Among the 28 cloned fragments cloned, 10 represent genes were previously reported (Table II). It is possible that upregulation of MHC and T cell antigen receptor molecules during the DTH reaction is responsible for their roles in the *in vivo* immune response. The elevated expression of the ribosomal RNA or protein and mitochondrion cytochrome *b* genes is probably an indicator of active metabolism and protein synthesis in the leukocytes infiltrating at the site of the DTH reaction. Of interest is that the expression of the ferritin heavy chain, elafin and Hox-1.7 genes was also upregulated. Due to its dual functions in iron detoxification and as an iron reserve, ferritin in monocytes/macrophages plays a key role in ferric iron metabolism, which is well known to be important in defensive responses to microorganism infections (*36*, *37*). Elafin, a recently found elastase-specific inhibitor, is predominantly produced by epithelial cells (*38*) and also exists in a small amount in neutrophils (*39*). Elafin and elastase have also been proposed to regulate blood vessel formation (*40*). Hox-1.7 is a guinea pig homeobox gene (*41*). In general, homeobox genes function as master genes in controlling tissue identity and pattern formation during ontogeny. Homeobox genes have been suggested to control leukomogenesis (*42*), and to have a proliferative effect on activated T cells (*43*) and NK cells (*44*). Nevertheless, it is not known whether or not they are involved in control of the *in vivo* immune response. The roles of the ferritin, elafin, and Hox-1.7 gene products in the DTH reaction need further investigation.

The screening of a guinea pig splenocyte-derived cDNA library with an insert of f120-c2 as a probe revealed that cloned f120-c2-5 is a 2,393-bp fragment with one possible open reading frame capable of encoding a peptide of 471 amino acid residues (Fig. 2). A homology search showed that this peptide is the guinea pig WRS cDNA because f120-c2-5 exhibits 84% homology with human and mouse WRS (Fig. 3). In particular, a C-terminal portion of guinea pig WRS, from residue 82 to the C-terminal end (refer to Fig. 3), exhibits 90% homology with them. In contrast, an insert of f120-c2 corresponds to a 3′-terminal of WRS cDNA (Fig. 2), and a 3′-untranslated region of WRS exhibits less homology than the coding region (*32*, *33*). This is why f120-c2 was first identified as a unique gene.

WRS, a house-keeping gene, was first found to be inducible by IFN by Fleckner *et al.* (*46*). WRS mRNA was shown to be strongly induced by IFN-γ in a human monocytic cell line, THP-1 (*47*), and to be involved in mononuclear phagocyte maturation (*48*). Xue and Wong (*49*) hypothesized that the induction of WRS might help in safeguarding tryptophan incorporation for IFN-enhanced synthesis of immunological molecules. It has been postulated that the Th1 cell is the "inducer" of the DTH response since it secretes IFN-γ (*50*). The findings that guinea pig WRS mRNA was expressed in macrophages and spleen cells, and that their expression was augmented by LPS or Con A therefore suggested that WRS may be induced by macrophages *via* IFN-γ. Further, WRS mRNA was highly expressed in SIC, although the amount of SIC RNA obtained was only approximately one-hundredth that of total skin RNA (data not shown). These results suggested that the expression of WRS was induced in infiltrating macrophages in association with the guinea pig DTH reaction. Determination of the biological significance of this induction awaits further study.

It has been reported that an alternatively spliced form of WRS mRNA was produced by the use of an alternative polyadenylation site in mouse and human (*33*, *45*). An alternatively spliced form of human WRS mRNA induced by IFN-γ possesses a shorter 3′-end than commonly expressed WRS mRNA (*45*). Our cloned guinea pig WRS cDNA corresponded to the commonly expressed mRNA in mouse and human, and an alternative polyadenylation sequence could not be found in the 3′-untranslated region. In agreement with this, only one band of guinea pig WRS mRNA was detected by Northern blot hybridization.

Recently, it was demonstrated that the C-terminal domain of mammalian tyrosyl-tRNA synthetase (YRS), which is not essential for aminoacylation function, exhibits the same cytokine activities as endothelial monocyte–activating polypeptide II (*51*). If WRS exhibits similar activities, the induction of WRS mRNA suggests that WRS itself may play an important role in the DTH reaction. It is worth investigating whether or not WRS has cytokine-like activities like YRS.

In this study, we identified a number of cDNA fragments that are upregulated in the DTH reactive skin tissue. Among them we cloned guinea pig WRS and revealed that after elicitation the upregulation of WRS was rather restricted to the skin. Additional studies are necessary to determine whether or not the elevated expression of these genes is critical for the DTH reaction.

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