American Journal of Pathology, Vol. 152, No. 6, June 1998

TRAF-4 Expression in Epithelial Progenitor Cells

Analysis in Normal Adult, Fetal, and Tumor Tissues

Maryla Krajewska,* Stanislaw Krajewski,* Juan M. Zapata,* Todd Van Arsdale,* Randy D. Gascoyne,† Ken Berern,‡ Deborah McFadden,§ Ahmed Shabaik,¶ Judith Hugh,‖ Carol Reynolds,** Charles V. Clevenger,** and John C. Reed*

From The Burnham Institute, La Jolla, and the Department of Pathology,¶ University of California, School of Medicine, San Diego, California; the British Columbia Cancer Agency,† Vancouver General Hospital,‡ and British Columbia Children's Hospital,§ Vancouver, British Columbia, and the Cross Cancer Institute,‖ Alberta, Canada; and the Department of Pathology and Laboratory Medicine,** University of Pennsylvania, Philadelphia, Pennsylvania*

TRAF-4 was discovered because of its expression in breast cancers and is a member of the tumor necrosis factor (TNF) receptor-associated factor (TRAF) family of putative signal-transducing proteins. *In vitro* binding assays demonstrated that TRAF-4 interacts with the cytosolic domain of the lymphotoxin-β receptor (LTβR) and weakly with the p75 nerve growth factor receptor (NGFR) but not with TNFR1, TNFR2, Fas, or CD40. Immunofluorescence analysis of TRAF-4 in transfected cells demonstrated localization to cytosol but not nucleus. Immunohistochemical assays of normal human adult tissues revealed prominent cytosolic immunostaining in thymic epithelial cells and lymph node dendritic cells but not in lymphocytes or thymocytes, paralleling the reported patterns of LTβR expression. The basal cell layer of most epithelia in the body was very strongly TRAF-4 immunopositive, including epidermis, nasopharynx, respiratory tract, salivary gland, and esophagus. Similar findings were obtained in 12- to 18-week human fetal tissue, indicating a highly restricted pattern of expression even during development. In the mammary gland, epithelial cells of the terminal ducts were strongly TRAF-4 immunopositive whereas myoepithelial cells and most of the mammary epithelial cells lining the extralobular ducts were TRAF-4 immunonegative. Of 84 primary breast cancers evaluated, only 7 expressed TRAF-4. Ductal carcinoma *in situ* (DCIS) lesions were uniformly TRAF-4 immunonegative (n = 21). In the prostate, the basal cells were strongly immunostained for TRAF-4, whereas the secretory epithelial cells were TRAF-4 negative. Basal cells in prostate hypertrophy (n = 6) and prostatic intraepithelial neoplasia (PIN; n = 6) were strongly TRAF-4 positive, but none of the 32 primary and 16 metastatic prostate cancer specimens examined contained TRAF-4-positive malignant cells. Although also expressed in some types of mesenchymal cells, these findings suggest that TRAF-4 is a marker of normal epithelial stem cells, the expression of which often ceases on differentiation and malignant transformation. *(Am J Pathol 1998, 152:1549–1561)*

The tumor necrosis factor (TNF) family of cytokine receptors plays important roles in host defenses against pathogens and immune responses to tumors.[1] Gene ablation studies indicate that some members of the TNF family of cytokines and cytokine receptors are critical for normal development of lymphoid organs or homeostasis of the immune system.[2,3] Although several TNF family receptors are expressed on epithelial cells, the body's first site of contact with most microorganisms and viruses, relatively little is known about the role of TNF family cytokines in epithelial cell biology.

Recently, a family of putative signal-transducing proteins has been identified that are collectively called TRAFs, TNF-receptor-associated factors. These proteins can associate with the cytosolic domains of certain members of the TNF receptor (TNFR) family, and some of them have been implicated in activation of NF-κB or other signaling pathways.[4–8] Membership in the TRAF family is predicated on the presence of a conserved ~150-amino-acid domain that mediates the interactions of these proteins with specific TNF family cytokine receptors, ie, the TRAF domain. To date, TRAF family proteins have generally been found within the cytosol of cells, sometimes in association with cytosolic vesicles, or at the plasma membrane after addition of appropriate TNF family cytokines to cells.[9–12]

Supported by CaP-CURE, the California Breast Cancer Research Program (2RB-0215) and the National Cancer Institute (CA-69381). J.M. Zapata is the recipient of a fellowship grant from the California Breast Cancer Research Program (3FB-0093). T. Van Arsdale is the recipient of a fellowship grant from the Leukemia Society of America (5253–97).

Accepted for publication March 13, 1998.

Address reprint requests to Dr. John C. Reed, The Burnham Institute, 10901 N. Torrey Pines Road, La Jolla, CA 92037. E-mail: jreed@burnham-institute.org.

TRAF-4 (also known as CART) is the only member of the TRAF family that was discovered initially for reasons unrelated to TNF family cytokine receptors. Rather, the TRAF-4 gene was discovered by differential screening of cDNA libraries in search for genes specifically expressed in malignant breast cancers.[13] The TRAF-4 gene maps to the chromosome 17 q11–21.3 region, which is amplified in up to 25% of adenocarcinomas of the breast. Moreover, the TRAF-4 protein has been reported to reside within the nuclei of malignant breast cells, suggesting that it might serve alternative functions compared with other TRAF family proteins.[6] In this report, we used an immunohistochemical approach to define the *in vivo* patterns of TRAF-4 expression in normal adult and fetal human tissues as well as in breast and prostate cancers.

Materials and Methods

Antiserum Preparation

A synthetic peptide NH_2-MPGFDYKFLEKPKRRLLC was conjugated to maleimide-activated carrier proteins (combination of ovalbumin and keyhole limpet hemocyanin (Pierce, Rockford, IL)) and used for immunization of a New Zealand White rabbit, essentially as described.[14]

Immunohistochemistry

Normal adult tissues for immunohistochemical analysis were derived from human biopsy and autopsy material from several individuals. Archival samples of human fetal tissue of 12 to 18 weeks gestation ($n = 4$) were obtained from the British Columbia Children's Hospital. The origin of the breast and prostate cancer specimens used here has been reported previously.[15,16] An additional 20 randomly chosen cases of lobular breast carcinoma were derived from patients presenting to the Cross Cancer Institute. Paraffin-embedded tissues were sectioned (5 μm) and immunostained using an ABC-diaminobenzidine-based detection method as described in detail.[14] Typically, the dilution of anti-TRAF-4 antiserum used was 1:500 or 1:1000 (v/v). Nuclei were counterstained with either hematoxylin or methyl green. For all tissues examined, the immunostaining procedure was performed in parallel using preimmune serum to verify specificity of the results. The antiserum was also preadsorbed with 5 to 10 μg/ml of the synthetic peptide immunogen, thus providing an additional control for immunospecificity. The immunostaining results were arbitrarily scored according to intensity as follows: 0, negative; 1+, weak; 2+, moderate; 3+, strong. Results presented for each tissue were based on immunohistochemical analysis of multiple immunostained slides. For dichotomizing breast and prostate cancer specimens into immunopositive and immunonegative groups, TRAF-4 immunostaining of 2+ to 3+ intensity was considered positive and 0 to 1+ was scored as negative. All TRAF-4-immunonegative tumor specimens evaluated contained TRAF-4-positive nonmalignant cells within the same sections that served as a control, verifying adequate preservation of the relevant TRAF-4 epitope and successful operation of the immunostaining procedure.

Immunoprecipitation Assays

TRAF-1-, TRAF-2-, TRAF-3-, TRAF-4-, TRAF-5-, and TRAF-6-encoding cDNAs were *in vitro* transcribed and translated using reticulocyte lysates (Promega, Madison, WI) containing [^{35}S]L-methionine as described.[14] The resulting *in vitro* translation mixes (4 μl) were diluted into ~0.15 ml of 20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 0.5% Nonidet P-40 with protease inhibitors, precleared, and then incubated with 1 μl of anti-TRAF-4 antiserum for 0.5 hour at 4°C. Immune complexes were collected by addition of 15 μl of 50% (v/v) protein G-Sepharose for 1 hour, washed extensively, and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

In Vitro *Protein Binding Assay Using Glutathione-S-Transferase (GST) Fusion Proteins*

GST fusion proteins containing the cytosolic domains of TNFR1, TNFR2, CD40, Fas, lymphotoxin-β receptor (LTβR), and p75 nerve growth factor receptor (NGFR) were produced in bacteria and affinity purified as described.[5,17] Approximately 5 μg of GST fusion proteins immobilized on 10 μl of Sepharose-glutathione were incubated for 1 hour at 4°C in 1 ml of binding buffer (20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 10% glycerol, 0.2% Triton X-100, 1 mmol/L dithiothreitol, 1 mmol/L phenylmethylsulfonyl fluoride, and 5 μg/ml pepstatin and leupeptin) with 5 μl for ^{35}S-labeled *in vitro* translated TRAF-4. After extensive washing, the beads were resuspended in Laemmli buffer and boiled, and the eluted proteins were analyzed by SDS-PAGE/autoradiography.

Immunofluorescence

293T cells were transiently transfected with either pcDNA3 control plasmid DNA or pcDNA-HA-TRAF-4, as previously described.[14] One or two days later, cells growing on coverslips were rinsed with PBS and fixed for 5 minutes at room temperature with Z-Fix solution (zinc-buffered formalin; Anatech, San Diego, CA). Thereafter, they were washed three times for 5 minutes with PBS before incubation with 20 mmol/L glycine or 0.1% sodium borohydrate in PBS for 15 minutes to quench autofluorescence, block free aldehyde groups, and enhance the antibody penetration. Coverslips were then incubated for 1 hour in preblocking solution containing 1% normal goat serum followed by incubation for 3 hours or overnight with anti-TRAF-4 antibody diluted 1:300 or 1:800 (v/v), respectively. After washing three times with PBS, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG was applied at 1:50 (v/v) for 1 hour. After washing three times, the specimens were counterstained with 1 μg/ml

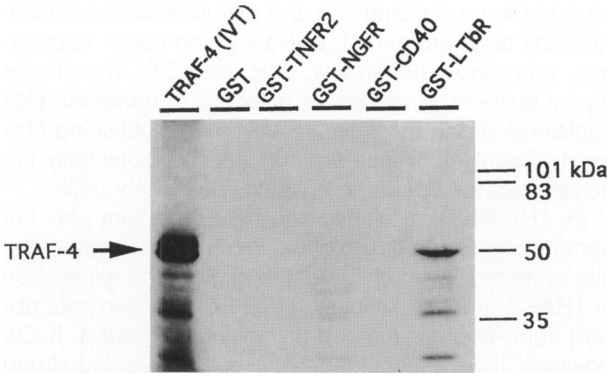


Figure 1. TRAF-4 binds *in vitro* to LTβR and NGFR. *In vitro* translated ^{35}S-labeled TRAF-4 was incubated with GST or various GST fusion proteins immobilized on glutathione-Sepharose. After extensive washing, proteins were eluted from beads in Laemmli solution and analyzed by SDS-PAGE/autoradiography. As a control, an equivalent amount of *in vitro* translated (IVT) ^{35}S-labeled TRAF-4 was loaded directly in the gel. In other experiments not shown here, TRAF-4 failed to bind GST-Fas or GST-TNFR1.

propidium iodide and covered with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Cells were photographed using a Nikon fluorescence microscope (Nikon UFX-IIA Optiphot).

Results

TRAF-4 Binds in Vitro *to LTβR and p75-NGFR*

With the exception of TRAF-4, all other TRAF family proteins have been shown to bind the cytosolic domains of specific members of the TNF cytokine receptor family. To preliminarily explore whether TRAF-4 potentially associates with TNF family receptors, ^{35}S-labeled *in vitro* translated TRAF-4 was incubated with GST fusion proteins representing the cytosolic domains of TNFR1, TNFR2, LTβR, Fas, CD40, and p75-NGFR. As shown in Figure 1, ^{35}S-labeled TRAF-4 clearly bound to GST-LTβR and also weakly but reproducibly reacted with GST-NGFR. TRAF-4, however, failed to bind TNFR1, TNFR2, Fas, or CD40. Experiments using various irrelevant ^{35}S-labeled non-TRAF-family proteins confirmed the specificity of these results. Thus, TRAF-4 potentially can interact with LTβR and possibly also with p75-NGFR.

Generation of TRAF-4-Specific Antiserum

A rabbit antiserum was generated using a synthetic peptide corresponding to amino acids 1 to 18 of the human TRAF-4 protein. This peptide was chosen in part for its lack of sequence homology to other TRAF family proteins. To determine whether the antiserum was specific for TRAF-4, cDNAs encoding TRAF-2, TRAF-3, TRAF-4, TRAF-5, and TRAF-6 were *in vitro* transcribed and translated in the presence of [^{35}S]L-methionine and immunoprecipitated with the anti-TRAF-4 peptide antiserum. As shown in Figure 2, only TRAF-4 was immunoprecipitated by this antiserum. Poor efficiency of *in vitro* translation of the TRAF-1 protein precluded analysis by this method, but FLAG-tagged TRAF-1 protein expressed in 293T cells

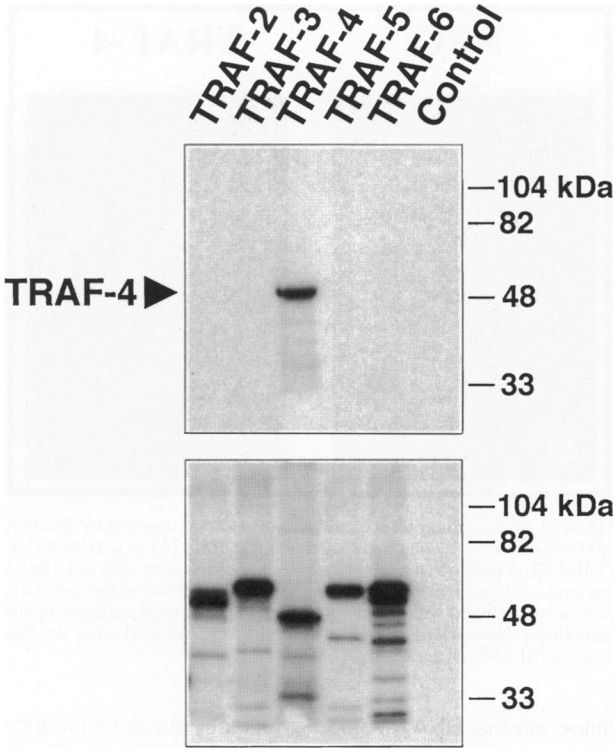


Figure 2. Anti-TRAF-4 antiserum specifically immunoprecipitates TRAF-4. TRAF family proteins TRAF-2, TRAF-3, TRAF-4, TRAF-5, and TRAF-6 were *in vitro* translated in the presence of [^{35}S]L-methionine and immunoprecipitated using anti-TRAF-4 antiserum (**A**) or analyzed directly by SDS-PAGE (**B**). A reticulocyte lysate primed with pcDNA-3 plasmid DNA was included as a control.

failed to immunoprecipitate with the anti-TRAF-4 peptide antiserum whereas a FLAG-TRAF-4 protein was successfully immunoprecipitated (not shown). Taken together, these results indicate that the anti-peptide antibody specifically recognizes TRAF-4 but not the other known members of the TRAF family. Although it reacted with TRAF-4 in immunoprecipitation assays, additional studies indicated that this anti-peptide antiserum does not work for immunoblotting.

Immunofluorescence Analysis of TRAF-4 in Transfected Cells

The subcellular location of the TRAF-4 protein was explored by immunofluorescence using 293T kidney epithelial cells that were transiently transfected with either an expression-plasmid-producing HA-epitope-tagged TRAF-4 protein or the same plasmid lacking a cDNA insert. Cells were counterstained with the DNA-binding fluorochrome propidium iodide to highlight the nucleus. Using the anti-TRAF-4 peptide antiserum, prominent immunofluorescence was detected within the cytosol of TRAF-4-transfected but not control-transfected 293T cells. The immunofluorescence was associated predominantly with cytosolic vesicles or organelles, similar to previous reports for the TRAF family proteins TRAF-1, TRAF-2, and TRAF-3.[9–12] These organelles apparently are not mitochondria, as propidium iodide staining of

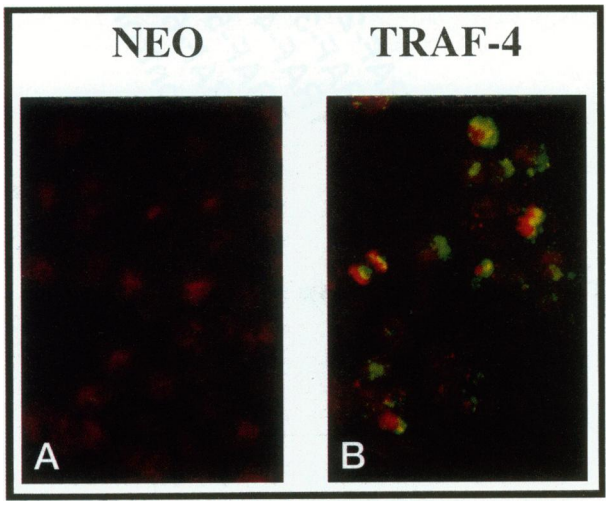


Figure 3. Immunofluorescence analysis of TRAF-4 intracellular location. 293T cells were transiently transfected with pcDNA-3 (**A**) or pcDNA-3-Flag-TRAF-4 (**B**). Two days later, cells were fixed and incubated with anti-TRAF-4 antiserum followed by FITC-conjugated goat anti-rabbit antiserum. Cells were counterstained with propidium iodide. Images were obtained by immunofluorescence microscopy. Similar results were obtained using anti-Flag monoclonal antibody (not shown).

mitochondrial DNA revealed a distinctly separate location for these organelles in the cytosol of 293T cells (Figure 3B), and two-color analysis using mitochondria-specific dyes failed to reveal co-localization of TRAF-4 with these organelles (not shown). Taken together, these immunofluorescence data further confirm the specificity of the anti-TRAF-4 antiserum and suggest that TRAF-4 is similar to other TRAF family proteins in its subcellular localization.

Immunolocalization of TRAF-4 in Normal Adult Human Tissues

Pilot experiments using paraffin-embedded 293T cells that had been transfected with a TRAF-4-producing expression plasmid indicated that the anti-peptide antiserum could recognize TRAF-4 protein in immunohistochemical assays (not shown). We therefore undertook an immunohistochemical survey of the *in vivo* patterns of TRAF-4 expression in normal adult human tissues. Table 1 summarizes the results, and Figure 4 presents some representative examples. In every case, the specificity of the TRAF-4 immunostaining results was confirmed through side-by-side comparisons of immune serum results with both preimmune serum and with TRAF-4 peptide-preabsorbed immune serum (not shown).

The most striking feature of TRAF-4 immunostaining was its strong presence in the basal cell layer lining the basement membrane of epithelia throughout the body. For example, the basal cell layer of keratinocytes within the epidermis and in the oropharynx contained strong cytosolic TRAF-4 immunoreactivity. Although some of the overlying differentiated cells also expressed TRAF-4, in general, the intensity of TRAF-4 immunostaining was markedly reduced in the upper layers of these and other complex epithelia (Figure 4, A–D). Similar observations were made for the stratified and pseudostratified epithelia of the respiratory tract, salivary gland ducts, esophagus, and cervix (Figures 4, E–H, and 2J). The simple ductal epithelia of the sweat glands and pancreas also contained strong cytosolic TRAF-4 immunostaining (Table 1; Figure 4I). These findings imply a potentially important role for TRAF-4 in epithelial progenitor cells.

As TNF family cytokines and their receptors play important roles in regulation of the immune and hematopoietic systems, it was of interest to explore the expression of TRAF-4 in these tissues. TRAF-4 expression was absent from lymphocytes and thymocytes (Figure 4, K–O). However, thymic reticuloepithelial cells contained strong cytosolic TRAF-4 immunoreactivity (Figure 4, K and L), and dendritic cells within lymph nodes were also TRAF-4 immunopositive (Figure 4O). Among the myeloid series of hematopoietic cells, mature granulocytes within peripheral blood and bone marrow were the most prominent expressors of TRAF-4 (Table 1). Monocytes, megakaryocytes, and erythroid precursors sometimes contained weak TRAF-4 immunostaining but were usually immunonegative.

As TRAF-4 weakly interacted with p75-NGFR, at least *in vitro*, we explored the expression of this TRAF family protein in the nervous system. As summarized in Table 1, little TRAF-4 expression was detected in the normal adult brain, spinal cord, or peripheral nervous system. Occasional large neurons within the basal ganglia, cerebellum (Purkinje cells), and dorsal root, cranial nerve, and autonomic ganglia contained weak or moderate (1+ to 2+) intensity TRAF-4 immunoreactivity in association typically with punctate cytosolic structures (Figure 4P). Interestingly, the axons of peripheral nerves were generally TRAF-4 immunopositive, raising the possibility that TRAF-4 may be transported to nerve termini rather than accumulating in the cell bodies of at least some types of neurons. Otherwise, little or no TRAF-4 expression was detected in the nervous system, except for occasional fibrillary reactive astrocytes.

Expression of TRAF-4 during Development

The analysis of TRAF-4 immunostaining in adult tissues suggested that this protein was prominently expressed in epithelial stem cells, which are derived from endoderm and ectoderm. To compare the expression of TRAF-4 in adult and developing tissues, human fetal tissues at 12 to 18 weeks gestation were analyzed by immunohistochemistry as described above. A brief description of the findings is presented here (data not shown).

Similar to adult tissues, strong cytosolic TRAF-4 immunostaining was found in the basal cells lining the basement membrane of the developing epithelia of endodermal and ectodermal origin. In the developing respiratory tract, for example, strong TRAF-4 immunostaining was present in the basal epithelial cells of the trachea and two main stem bronchi. However, TRAF-4 immunoreactivity was not evident in the single-cell layer of epithelial cells lining the smaller bronchioles or in the mesenchyme-derived alveolar cells. Some other types of cells of

Table 1. TRAF-4 Immunoreactivity in Normal Human Tissues

Organ/tissue	Structure/cell type	Intensity
Skin		
Epidermis	Keratinocytes	
Stratum basale	Basal cell layer	3
Stratum spinosum	Spinous layer	1–2
Stratum granulosum	Granular layer	1–2
Stratum corneum	cornified layer	0–2
Dermis		
Connective stroma	Fibroblasts	0
Sweat gland	Epithelium	2–3
Sebaceous gland	Epithelium	0
Musculoskeletal system		
Skeleton		
Cartilage	Chondrocytes	1–3
	Fibroblasts (fibrocartilage)	0
Bone	Osteocytes	0
	Osteoclasts	0
Striated Muscles	Muscle fibers	0–1
Cardiovascular system		
Heart		
Myocardium:	Myocytes	0–1
	Fibroblasts	0–1
	Capillary endothelium	0
Arteries	Endothelial cells	0
	Smooth muscle cells	1–3
	Fibroblasts	0
Respiratory system		
Trachea		
Epithelium (pseudostratified columnar)	Basal cell layer	2–3
	Luminal cell layer	0–1
Submucosa	Fibroblasts	0
	Smooth muscle cells	0–1
	Sero-mucous glands	0
Cartilage	Chondrocytes	0
Lung		
Bronchi (pseudostratified or simple columnar epithelium)	Basal layer	2–3
	Luminal layer	0
Alveoli	Type I pneumocytes	0
	Type II pneumocytes	0
	Alveolar macrophages	0
Alimentary tract		
Salivary gland (submandibular gland)		
Secretory gland acini	Serous cells	0
	Mucous cells	0
Excretory duct epithelium	Basal cells	2–3
	Luminal layer	1
Tongue/esophagus		
Stratified squamous epithelium	Basal cell layer	3
	Spinous layer	0–1
	Granular layer	1–2
Muscularis externa	Smooth muscle cells	0–1
Stomach		
Cardiac region	Gastric pits/foveolar cells	0–2
	Cardiac glands/mucoid cells	0
Submucosal plexus (Meissner's plexus)	Ganglion cells	0
	Smooth muscle cells	0–1
Small intestine	Absorptive epithelium	0
	Paneth cells	0
Colon	Absorptive cells	0–2*
	Goblet/mucous cells	0–2
Myenteric plexus (Auerbach's plexus)	Ganglion cells	0
Liver	Hepatocytes	0–1
	Sinusoidal endothelium	0
	Bile duct epithelium	0–2
Pancreas		
Exocrine	Acinar cells	0
	Ductal epithelium	2–3
Endocrine: islets of Langerhans		0

Table 1. Continued

Organ/tissue	Structure/cell type	Intensity
Urinary system		
Kidney		
Glomeruli	Mesangial cells	0
Bowman's capsule	Parietal layer/podocytes	0
	Visceral layer/squamous epithelium	0
Collecting tubules	Proximal convoluted tubules	1–2
	Loop of Henle, thin limb	0
	Distal convoluted tubules	1–2
Collecting ducts	Epithelial cells	2
Urinary bladder	Transitional epithelium	0–1
	Smooth muscle cells	0–1
Male reproductive systems		
Testis	Leydig cells	1–2
Seminiferous tubules	Spermatogonia	0
	Sertoli cells	0
	Spermatocytes	1–3
	Spermatids	0
	Spermatozoa	1–2
Vas Deferens	Pseudostratified columnar epithelium	1–2
Prostate		
Tubuloalveolar glands	Basal cells	1–3
	Luminal secretory cells	0
Fibromuscular stroma	Smooth muscle cells	0–2
	Fibroblasts	0
Female reproductive system		
Vagina		
Epithelium	Basal cell layer	3
	Spinous layer	1–2
	Granular layer	1–2, sc 3
Uterus		
Endometrium	Columnar epithelium	0–1
	Stromal cells	0–1
Myometrium	Smooth muscle cells	0–1
Oviduct (Fallopian tube)		
Tunica mucosa	Ciliated columnar epithelium	1–2
	Secretory cells	0–2
Tunica muscularis	Smooth muscle cells	0–1
Mammary gland		
Tubuloalveolar glands	Cuboidal/columnar epithelium	2–3
Lactiferous ducts	Columnar epithelium	0–1
	Myoepithelial cells	0
Loose/fibrous stroma	Fibroblasts	0
Hematolymphoid system		
Peripheral blood	Granulocytes	2–3
	Monocytes	0–1
	Lymphocytes	0
	Erythrocytes	0
Bone marrow	Erythroid precursors	0–1
	Myeloid precursors	0
	Megakaryocytes	0–1
	Mature neutrophils	0–3
	Plasma cells	0
	Monocytes	0–1
Thymus		
Cortex	Cortical thymocytes	0
	Macrophages (dendritic interdigitating cells)	0
Medulla	Epitheliorecticular cells	3
	Hassall's corpuscles	1–3
	Medullary thymocytes	0
Palatine tonsil		
Stratified squamous epithelium	Basal cell layer	3
	Spinous cell layer	1
	Granular cell layer	1–2

Table 1. Continued

Organ/tissue	Structure/cell type	Intensity
Tonsil/Lymph nodes		
Germinal center	Large noncleaved cells	0
	Small noncleaved cells	0
	Small cleaved cells	0
	Follicular dendritic cells	0
	Macrophages	0
Mantle zone	Lymphocytes	0
Interfollicular region	Small T cells	0
	Large transformed cells	0
	Sinus histiocytes	0–2
	Plasma cells	0, sc 2
Spleen		
Periarteriolar sheets		
Follicles	T-Lymphocytes	0
Marginal zone	B-Lymphocytes	0
Red pulp	B-Lymphocytes	0
	Spleen cords	0
	Sinus lining cells	0
Central nervous system		
Brain		
Gray matter	Normal neurons	0, sc 2
	Axons	0
	Neurophil	0
White matter	Myelin sheath	0
Neuroglia	Astrocytes	0–(2†)
	Oigodendroglia	0
Microglia		0
Ependyma	Ependymal cells	0–1
Leptomeninges	Arachnoid epithelium	0
Choroid plexus	Epithelium	0
Spinal cord		
White matter	Axons	0–1
	Myelin sheath	0
	Astroglia	0–(2†)
Gray matter	Ventral horn motoneurons	0
	Dorsal horn sensory neurons	0–1
	Neuropil	0
Central channel	Ependyma	0–1
Peripheral nervous system		
Ganglia	Neurons	0–1
	Satellite cells	0
	Schwann cells	0
	Fibroblasts	0
Peripheral nerves	Axon	1–3
	Myelin sheath	0
Endocrine system		
Thyroid		0
Adrenal		0

sc, occasional scattered cells.
* supranuclear organellar-like staining.
† Only fibrillary astrocytes are positive.

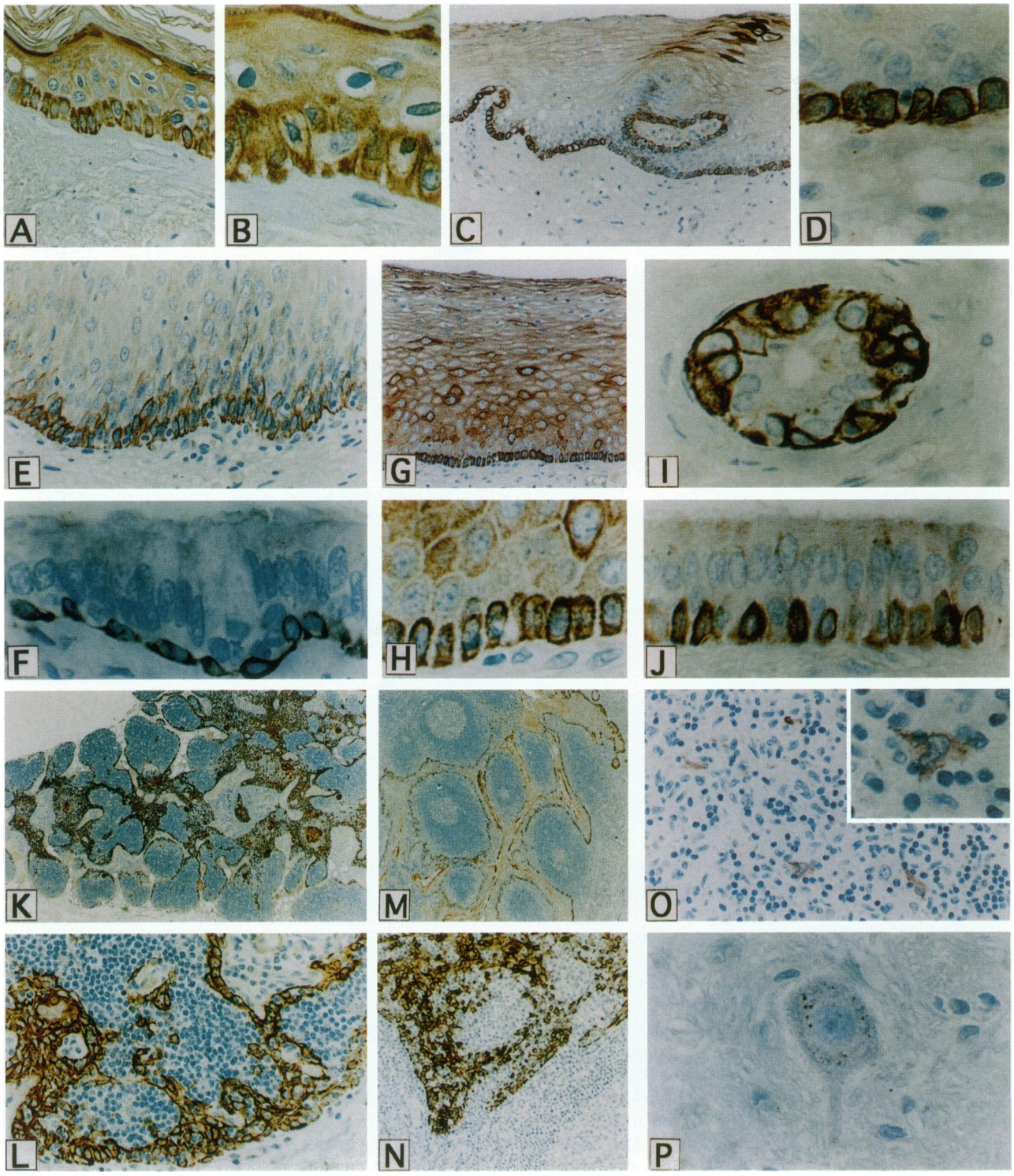
endodermal origin, such as hepatocytes and thymic epithelium, were also TRAF-4 immunopositive, whereas TRAF-4 expression was not evident in most mesenchymal cells, including developing hematopoietic cells in the fetal liver and thymocytes. Despite its origin from ectoderm, relatively little TRAF-4 expression was found in the developing nervous system. For example, in the developing cerebral cortex, only occasional TRAF-4-immunopositive neural precursors were observed along the radial glia processes that guide the migration of these cells. In the developing spinal cord, TRAF-4 expression was limited to occasional astrocytes. Dorsal root ganglion neurons, however, uniformly contained faint (1+) TRAF-4 immunostaining. Thus, highly restricted patterns of TRAF-4 expression were observed during fetal development, which were similar in most cases to those seen in adult human tissues of endodermal and ectodermal origin.

Expression of TRAF-4 in Normal and Neoplastic Mammary Epithelium

As TRAF-4 expression has been reported in breast cancers,[6,13] we evaluated by immunohistochemistry the expression of TRAF-4 in 84 cases of breast cancer and compared the results with normal mammary epithelium. These breast cancers included 58 infiltrating ductal car-

cinomas (16 well differentiated and 42 poorly differentiated), 23 lobular, 1 medullary, 1 fibrosarcomatous, and 1 adenoid cystic cancer. In addition, 3 ductal carcinoma *in situ* (DCIS) specimens were also evaluated, along with the DCIS components that co-existed with invasive cancer in 18 of the tumor specimens (total $n = 21$). Assessment of normal mammary epithelium was made from 3 reduction mammoplasty specimens and residual histologically normal mammary gland epithelium that was present within 39 of the tumor specimens (total $n = 42$).

In normal mammary gland tissue, strong cytosolic TRAF-4 immunostaining was present in the epithelial cells of the terminal ducts of the lobular units, where the highest rates of cell proliferation occur in the adult breast.[18] The underlying myoepithelial cells, however, were TRAF-4 negative (Figure 5, A and B). Moreover, the co-



lumnar mammary epithelial cells of the extralobular ducts were usually TRAF-4 negative (Figure 5C).

Most invasive breast cancers (77/84, 92%) and all cases of DCIS (21/21, 100%) were either entirely negative for TRAF-4 or only weakly immunostained. Figure 5, D and E, shows examples of DCIS in which the neoplastic epithelial cells exhibit only faint TRAF-4 immunoreactivity, but residual nests of non-neoplastic mammary epithelial cells contain intense TRAF-4 immunostaining. An example of an invasive cancer is shown in Figure 5F, with the overlying epidermis serving as a positive control by virtue of the strongly TRAF-4-positive keratinocytes. Only 7 of the 84 invasive cancers (<10%) contained malignant cells with moderate to strong TRAF-4 immunoreactivity, including 5 well differentiated infiltrating ductal, 1 poorly differentiated infiltrating ductal, and 1 adenoid cystic carcinoma. The TRAF-4 immunostaining was consistently present within the cytosol of these malignant breast cancer cells. Lobular carcinomas were uniformly TRAF-4 immunonegative (n = 23).

TRAF-4 Expression in Normal and Malignant Prostate Gland

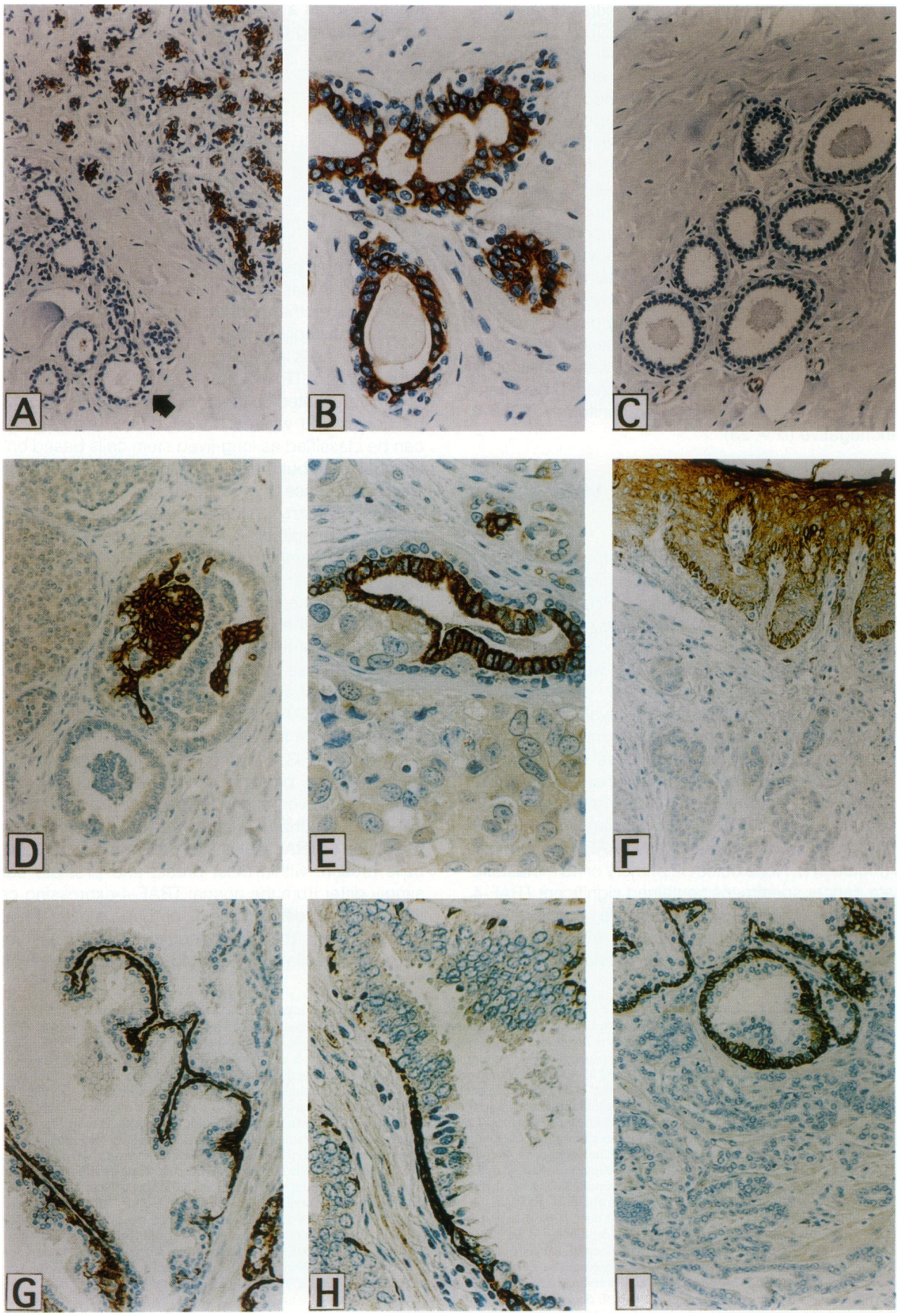
TRAF-4 expression was evaluated by immunohistochemistry in several normal prostate specimens, 6 biopsies each of benign prostatic hyperplasia (BPH) and prostatic intraepithelial neoplasia (PIN), 32 primary prostate adenocarcinomas, 8 metastases to lymph nodes, and 8 metastases to bone marrow. Because PIN was found adjacent to invasive cancer in several of the tumor specimens, 17 total PIN lesions were evaluated. In normal prostate glands, strong TRAF-4 immunoreactivity was present within the cytosol of the basal cells lining the basement membrane whereas the luminal secretory epithelial cells were either entirely TRAF-4 immunonegative or only faintly stained (Figure 5G). Similarly, in BPH and PIN, only the basal cells were clearly TRAF-4 immunopositive (Figure 5H). None of the 32 primary or 16 metastatic prostate cancer specimens contained significant TRAF-4 immunostaining. Figure 5I, for example, shows a well differentiated TRAF-4-negative tumor, with co-existing hyperplastic and normal-appearing prostatic epithelium that served as a positive control because of the strong immunostaining of the basal cells.

Discussion

By generating a specific anti-peptide antiserum that recognizes TRAF-4, we have explored the *in vivo* patterns of expression of this TRAF family protein in normal adult and developing human tissues using an immunohistochemical approach. Expression of TRAF-4 was highly restricted. The most prominent immunostaining for TRAF-4 was found in basal cells lining the basement membrane of complex epithelium throughout much of the body. This observation implies that TRAF-4 is expressed in epithelial progenitor cells and declines on induction of epithelial differentiation. TRAF-4 thus may serve as a novel marker of undifferentiated epithelial progenitor cells. It remains to be determined whether these TRAF-4-expressing cells can be classified as long-lived stem cells based on functional criteria, but their anatomical location suggests this may be the case.

Although many epithelial cancers are believed to be derived from epithelial stem cells, expression of TRAF-4 was rare in primary breast cancers (<10%) and undetectable in either primary or metastatic prostate cancers, in contrast to the strong TRAF-4 expression in the normal mammary epithelial cells located in the terminal ductlobular units of the breast and in the basal cells of the prostate. These observations raise the question of whether breast and prostate cancers down-regulate expression of TRAF-4 as part of the malignant transformation process. In this regard, some TRAF family proteins, such as TRAF-3, may convey signals from TNF family receptors that promote cell cycle arrest and apoptosis.[17,19] However, a phenotype for TRAF-4 has thus far not been described. Rather than a pathological down-regulation of TRAF-4, it is also possible that the malignant cells present within most breast and prostate cancers simply differ from the normal TRAF-4-expressing cells in their state of differentiation, perhaps similar to normal epithelial cells that down-regulate TRAF-4 as they undergo differentiation. It should be noted, however, that

Figure 4. Immunohistochemical analysis of TRAF-4 expression in normal adult tissues. Representative examples of TRAF-4 anti-peptide immunostaining results are presented. Immunostaining was accomplished by a diaminobenzidine detection method (brown color), and nuclei were counterstained with hematoxylin (blue). **A** and **B:** Epidermis, showing prominent cytosolic TRAF-4 immunoreactivity in the basal layer of keratinocytes, with diminished expression in the differentiated cells in the upper layers of the epithelium at ×200 and ×1000 original magnification, respectively. In some areas of skin, the cells of the uppermost layer (stratum granulosum) before the acellular cornified layer (stratum corneum) also were TRAF-4 immunopositive. **C** and **D:** Epithelium overlying the tongue at ×150 and ×1000, showing strong cytosolic TRAF-4 immunoreactivity in the basal cell layer of keratinocytes lining the basement membrane. Only occasional differentiated epithelial cells in the upper layer of the epithelium expressed TRAF-4. **E:** Esophagus, demonstrating TRAF-4 immunopositivity of the basal cells along the basement membrane and little or no TRAF-4 expression in the overlying differentiated cells of this stratified squamous epithelium. Magnification, ×200. **F:** Bronchial epithelium, showing TRAF-4 expression limited to the cells lining the basement membranes in this pseudostratified epithelium. Magnification, ×1000. **G** and **H:** Uterine cervix, demonstrating strong TRAF-4 immunostaining in the cytosol of the cells along the basement membrane, with weak to moderate TRAF-4 immunostaining in most of the differentiated cervical epithelial cells in this stratified squamous epithelium. Magnification, ×150 and ×1000. **I:** Pancreatic duct at ×1000 magnification showing strong cytosolic TRAF-4 immunostaining in the cells adjacent to the basement membrane, with little or no TRAF-4 immunoreactivity seen in the cells closest to the lumen. **J:** Salivary gland duct, demonstrating TRAF-4 expression in most of the basal cells along the basement membrane and only weak TRAF-4 immunoreactivity in the upper layers of cells closer to the lumen. Magnification, ×1000. **K** and **L:** Thymus, at ×50 and ×400 magnification, showing strong cytosolic TRAF-4 immunostaining in the reticuloepithelial cells but absence of TRAF-4 in the thymocytes. **M** and **N:** Tonsil, showing secondary lymphoid follicules amid invaginations of the overlying nasopharyngeal epithelium at ×80 and ×200 magnification. Note that the lymphoid cells within the secondary follicles are immunonegative. The undifferentiated keratinocytes (basal cells) contain strong cytosolic TRAF-4 immunoreactivity, whereas the differentiated keratinocytes are weakly stained for TRAF-4. **O:** Lymph node at ×400 magnification showing TRAF-4 immunostaining only in what appear to be dendritic cells, whereas lymphocytes and histiocytes are immunonegative. The inset represents a ×1000 photomicrograph of a TRAF-4-immunopositive dendritic cell. **P:** Brain, demonstrating an example of a large neuron in the reticular formation of the brain stem that contains cytosolic structures exhibiting TRAF-4 immunopositivity within the perikaryon. The surrounding glial cells are immunonegative. Magnification, ×1000.


A
B
C
D
E
F
G
H
I

the breast and prostate cancers evaluated here included many that were characterized as high-grade poorly differentiated tumors. Moreover, although it could be argued that many such tumors contain only a subpopulation of truly clonigenic stem cells, we failed to detect TRAF-4 immunostaining in even 1% of the tumor cells within cancer specimens that were scored as TRAF-4 immunonegative.

The TRAF-4 gene maps to the 17 q11–21.3 region, which harbors the c-erbB2 and BRCA1 genes and which is commonly amplified in breast carcinomas.[13] However, analysis of the TRAF-4 gene failed to demonstrate increased copy number in tumors that contained amplification of other 17q genes. Moreover, it is unclear to what extent contaminating nonmalignant TRAF-4-expressing cells may have contributed to the presence of TRAF-4 mRNA in breast cancer specimens. The advantage of the immunohistochemical approach taken here is that it permits direct localization of the cells that express TRAF-4 within the complex mixtures of normal and malignant cells that constitute most clinical biopsy specimens. Based on our findings obtained with 84 cases of primary breast cancer, TRAF-4 appears to be expressed at significant levels in only a small proportion (<10%) of these tumors. Studies of TRAF-4 expression in metastatic breast cancer samples, however, are required to determine whether changes in TRAF-4 are associated with progression from locally invasive to metastatic disease.

The cytosolic location of TRAF-4 immunostaining observed using the anti-peptide antiserum described here resembles that described for other TRAF family proteins. Moreover, in transfection experiments, similar results were obtained for FLAG-epitope-tagged TRAF-4, using anti-FLAG monoclonal antibody for immunolocalization studies (unpublished observations). TRAF-1, TRAF-2, and TRAF-3 have been localized by immunofluorescence to the cytosol of cells, sometimes in association with as yet unidentified cytosolic vesicles or organelles. After addition of appropriate TNF family ligands or expression of active receptors, these TRAF family proteins can be recruited to the plasma membrane, presumably through their inducible interactions with the cytosolic domains of particular TNF family cytokine receptors or viral proteins that mimic the actions of these receptors, such as the LMP-1 protein of Epstein-Barr virus.[9–12] A survey of TRAF-3 immunostaining in normal adult human tissues has also demonstrated cytosolic immunolocalization in the preponderance of cells throughout the body that express that member of the TRAF family.[14] Our findings, however, differ from a previous report that used a polyclonal antiserum raised against the TRAF-4 protein where nuclear immunostaining was observed.[6] The reason for the difference in these results is unclear, particularly as few details were provided about the specificity of that antiserum. It is formally possible, however, that different versions of the TRAF-4 protein can be produced through alternative mRNA splicing or proteolytic processing, for example, with our anti-peptide antibody recognizing only some forms of this protein.

The significance of the *in vitro* interactions of TRAF-4 with LTβR and NGFR remains to be determined, but these observations suggest that TRAF-4 most likely functions within the signal transduction pathways regulated by TNF family cytokine receptors, akin to other members of the TRAF family. Although many details about the expression of LTβR remain unclear, it has been reported that T and B lymphocytes fail to express this member of the TNFR family whereas thymic stroma and follicular dendritic cells do express LTβR.[20] The patterns of LTβR expression therefore parallel those seen here for TRAF-4, lending support to the idea that TRAF-4 could participate in LTβR signaling mechanisms *in vivo*. Although gene knockout experiments involving the LTβR gene have not been described to date, some functions of LTβR can potentially be inferred from experiments where either a LTβR-Fc fusion protein was expressed in transgenic mice, acting as a decoy for LTβR-binding ligands, or where the genes encoding ligands for LTβR were ablated in mice.[21–25] In this regard, cell surface LTα/LTβ heterotrimers form a high-affinity ligand for LTβR, whereas LTα homotrimers fail to bind and LTβ homotrimers fail to form.[20] Targeted disruption of the LTα and LTβ genes results in developmental abnormalities in lymphoid organogenesis and cytoarchitecture, with complete or partial loss of peripheral lymph nodes, defects in Peyer's patch formation, absence of germinal centers, and disorganization of the normal T cell and B cell regions of the spleen.[21–25] The thymus glands of these animals, however, are purportedly normal, without imbalances in the relative ratios of various T cell subsets. It remains to be determined whether and how the expression of TRAF-4 in thymic epithelial cells and lymph node dendritic cells functionally relates to the phenotypes of these animals with defects in LTα or LTβ production, but the expression of TRAF-4 in these scaffold cells is consistent with the reported role for LTα and LTβ in creation of tissue microenvironments suitable for immune cell interactions.

Figure 5. TRAF-4 expression in normal and malignant breast and prostate. **A** to **C**: Normal mammary epithelium, showing strong TRAF-4 immunostaining in the luminal epithelial cells of interlobular terminal ducts (acini) but absence of TRAF-4 in myoepithelial cells along the basement membrane (**A** and **B**). The epithelial cells lining the collecting ducts (arrow) are mostly TRAF-4 immunonegative (**A** and **C**). Magnification, ×100 (**A**), ×400 (**B**), and ×200 (**C**). **D**: DCIS, showing several ducts filled with carcinoma cells. An example of a solitary duct that contained residual TRAF-4-expressing normal acinar epithelial cells is shown, mixed with neoplastic cells. Magnification, ×200. **E**: Invasive breast cancer, demonstrating weak TRAF-4 immunostaining in the invasive carcinoma cells. The carcinoma appears to be invading a normal acinar duct that contains a layer of TRAF-4-immunopositive luminal epithelial cells and TRAF-4-immunonegative myoepithelial cells along the basement membrane. Magnification, ×400. **F**: Invasive breast cancer, showing TRAF-4-immunonegative carcinoma cells with overlying epidermis that contains TRAF-4-immunostained keratinocytes. Magnification, ×100. **G**: Normal prostate epithelium, showing prominent TRAF-4 immunostaining of basal cells along the basement membrane and absence of staining in the luminal secretory cells. Magnification, ×400. **H**: PIN, demonstrating TRAF-4-immunopositive basal cells along basement membrane with overlying epithelial cells exhibiting either weak or no TRAF-4 immunoreactivity. Magnification, ×400. **I**: Invasive prostate cancer (Gleason grade 2 to 4), showing absence of TRAF-4 immunostaining in carcinoma (bottom) but strong TRAF-4 immunostaining in basal cells of residual nonmalignant epithelium (top). Magnification, ×200.

In contrast to LTβR, the *in vivo* patterns of the expression of the p75 subunit of the NGFR have been studied extensively, revealing expression on most peripheral and some central neurons and some types of epithelial cells, among other cell types.[26–28] Only occasional neuronal cells appeared to express TRAF-4, both in fetal and adult tissues, suggesting that p75-NGFR/TRAF-4 interactions may be of limited relevance for these cells. It should be noted, however, that the unique cytoarchitecture of neurons, with their long axonal processes that transport NGF/p75 NGFR complexes retrograde from synaptic termini to the perikaryon, may complicate attempts to discern relations between TRAF-4 and NGFR expression. In contrast, expression of p75-NGFR has been reported in epithelial cells of the prostate and some basal cell keratinocytes,[29,30] which also express TRAF-4. Moreover, expression of p75-NGFR is commonly lost in prostate cancers,[29,31] similar to our observations for TRAF-4. The significance of this loss of p75-NGFR may be attributable to the ability of this receptor to stimulate apoptosis when not occupied by its ligand.[32]

In summary, the *in vivo* patterns of TRAF-4 expression suggest that this protein is produced in a highly tissue-restricted manner, with epithelial basal cells representing the most prominent expressors of this TRAF family protein. TRAF-4 thus may serve as a novel marker for analysis of epithelial stem cells. Although expressed in normal terminal duct mammary epithelial cells and basal cells of the prostate, TRAF-4 is only rarely expressed in breast and prostate cancers. Along with other established biomarkers, such as cytokeratins, therefore, immunostaining for TRAF-4 potentially may be of diagnostic assistance when differentiating benign hypertrophic or dysplastic conditions from malignant cancer. Finally, the potential interaction and co-expression of TRAF-4 with LTβR deserves further evaluation but suggests that TRAF-4 may participate in signal transduction pathways used by this member of the TNFR family.

Acknowledgments

We thank M-C. Rio and C. H. Regnier for the TRAF-4 cDNA, C. Care for GST-LTβR, D. Bredesen for GST-NGFR, J. Epstein for prostate cancer specimens, and H. Gallant and K. Le for manuscript preparation.

References

1. Bazzoni F, Beutler B: The tumor necrosis factor ligand and receptor families. N Engl J Med 1996, 334:1717–1725
2. Liu YJ, Banchereau J: Mutant mice without B lymphocyte follicles. J. Exp Med 1996, 184:1207–1211
3. Nagata S: Fas-induced apoptosis and diseases caused by its abnormality. Genes Cells 1996, 1:873–879
4. Rothe M, Wong SC, Henzel WJ, Goeddel DV: A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kd tumor necrosis factor receptor. Cell 1994, 78:681–692
5. Sato T, Irie S, Reed JC: A novel member of the TRAF family of putative signal transducing proteins binds to the cytosolic domain of CD40. FEBS Lett 1995, 358:113–118
6. Régnier CH, Tomasetto C, Moog-Lutz C, Chenard M-P, Wendling C, Basset P, Rio M-C: Presence of a new conserved domain in CART1, a novel member of the tumor necrosis factor receptor-associated protein family, which is expressed in breast carcinoma. J Biol Chem 1995, 270:25715–25721
7. Nakano H, Oshima H, Chung W, Williams-Abbott L, Ware CF, Yagita H, Okumura K: TRAF5, an activator of NF-kappaB and putative signal transducer for the lymphotoxin-β receptor. J Biol Chem 1996, 271: 14661–14664
8. Ishida T, Mizushima S-i, Azuma S, Kobayashi N, Tojo T, Suzuki K, Aizawa S, Watanabe T, Mosialos G, Kieff E, Yamamoto T, Inoue J-i: Identification of TRAF6, a novel tumor necrosis factor receptor-associated factor protein that mediates signaling from and amino-terminal domain of the CD40 cytoplasmic region. J Biol Chem 1996, 271: 28745–28748
9. Mosialos G, Birkenback M, Yalamanchili R, Van Arsdale T, Ware C, Kieff E: The Epstein-Barr virus transforming protein LMP1 engages signaling proteins for the tumor necrosis factor receptor family. Cell 1995, 80:389–399
10. Devergne O, Hatzivassiliou E, Izumi KM, Kaye KM, Kleijnen MF, Kieff E, Mosialos G: Association of TRAF1, TRAF2, and TRAF3 with an Epstein-Barr virus LMP1 domain important for B-lympyhocyte transformation: role in NF-κB activation. Mol Cell Biol 1996, 16:7098–7108
11. Van Arsdale TL, Van Arsdale SL, Force WR, Walter BN, Mosialos G, Keith E, Browning JL, Reed JC, Ware CF: Lymphotoxin-β receptor signaling complex: role of tumor necrosis factor receptor-associated factor 3 recruitment in cell death and activation of nuclear factor κB. Proc Natl Acad Sci USA 1997, 94:2460–2465
12. Lee SY, Lee SY, Kaudata G, Liou M-L, Liou H-C, Choi Y: CD30/TNF receptor-associated factor interaction: NF-kB activation and binding specificity. Proc Natl Acad Sci USA 1996, 93:9699–9703
13. Tomasetto C, Régnier C, Moog-Lutz C, Mattei MG, Chenard MP, Lidereau R, Basset P, Rio MC: Identification of four novel human genes amplified and overexpressed in breast carcinoma and localized to the q11–q21.3 region of chromosome 17. Genomics 1995, 28:367–376
14. Krajewski S, Zapata JM, Krajewski M, Van Arsdale T, Shabaik A, Gascoyne RD, Reed JC: Immunohistochemical analysis of *in vivo* patterns of TRAF-3 expression, a member of the TNF receptor-associated factor family. J Immunol 1997, 159:5841–5852
15. Reynolds C, Montone KT, Powell CM, Tomaszewski JE, Clevenger CV: Distribution of prolactin and its receptor in human breast carcinoma. Endocrinology 1997, 138:5555–5560
16. Krajewska M, Krajewski S, Epstein JI, Shabaik A, Sauvageot J, Song K, Kitada S, Reed JC: Immunohistochemical analysis of bcl-2, bax, bcl-X, and mcl-1 expression in prostate cancers. Am J Pathol 1996, 148:1567–1576
17. Russo J, Calaf G, Russo IH: Influence of age and gland topography on cell kinetics of normal human breast tissue. J Natl Cancer Inst 1987, 78:413–418
18. Eliopoulos AG, Dawson CW, Mosialos G, Floettmann JE, Rowe M, Armitage RJ, Dawson J, Zapata JM, Kerr DJ, Wakelam MJ, Reed JC, Kieff E, Young LS: CD40-induced growth inhibition in epithelial cells is mimicked by Epstein-Barr virus-encoded LMP1: involvement of TRAF3 as a common mediator. Oncogene 1996, 13:2243–2254
19. Ware CF, Abbott LW, Force WR, Mauri D: Lymphotoxin-β. J Immunol 1996, 124–148
20. Matsumoto M, Mariathasan S, Nahm MH, Baranyay F, Peschon JJ, Chaplin DD: Role of lymphotoxin and the type I TNF receptor in the formation of germinal centers. Science 1996, 271:1289–1291
21. Togni PD, Goellner J, Ruddle NH, Streeter PR, Fick A, Mariathasan S, Smith SC, Carlson R, Shornick LP, Schoenberger JS, Russell JH, Karr R, Chaplin DD: Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. Science 1994, 264:703–706
22. Koni PA, Sacca R, Lawton P, Browning JL, Ruddle NH, Flavell RA: Distinct roles in lymphoid organogenesis for lymphotoxins α and β revealed in lymphotoxin β-deficient mice. Immunity 1997, 6:491–500
23. Ettinger R, Browning JL, Michie SA, van Ewijk W, McDevitt HO:

Disrupted splenic architecture, but normal lymph node development in mice expressing a soluble lymphotoxin-β receptor-IgG1 fusion protein. Proc Natl Acad Sci USA 1996, 93:13102–13107
24. Matsumoto M, Lo SF, Carruthers CJL, Min J, Mariathasan S, Huang G, Plas DR, Martin SM, Geha RS, Nahm MH, Chaplin DD: Affinity maturation without germinal centres in lymphotoxin-α-deficient mice. Nature 1996, 382:462–466
25. Page KJ, Everitt BJ: The distribution of neurons coexpressing immunoreactivity to AMPA-sensitive glutamate receptor subtypes (GluR1–4) and nerve growth factor receptor in the rat basal forebrain. Eur J Neurosci 1995, 7:1022–1033
26. Chiu AY, Chen EW, Loera S: A motor neuron-specific epitope and the low-affinity nerve growth factor receptor display reciprocal patterns of expression during development, axotomy, and regeneration. J Comp Neurol 1993, 328:351–363
27. Pincelli C, Fantini F, Giannetti A: Nerve growth factor and the skin. Int J Dermatol 1994, 33:308–312
28. Perez M, Regan T, Pflug B, Lynch J, Djakiew D: Loss of low-affinity nerve growth factor receptor during malignant transformation of the human prostate. Prostate 1997, 30:274–279
29. Redd PE, Byers MR: Regeneration of junctional epithelium and its innervation in adult rats: a study using immunocytochemistry for p75 nerve growth factor receptor and calcitonin gene-related peptide. J Periodont Res 1994, 29:214–224
30. Pflug BR, Dionne C, Kaplan DR, Lynch J, Djakiew D: Expression of a Trk high affinity nerve growth factor receptor in the human prostate. Endocrinology 1995, 136:262–268
31. Rabizadeh S, Oh J, Zhong L, Yang J, Bilter C, Butcher L, Bredesen D: Induction of apoptosis by the low-affinity NGF receptor. Science 1993, 261:345–348