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Tumorigenesis and Neoplastic Progression

A Novel Aspartic Proteinase-Like Gene Expressed in Stratified Epithelia and Squamous Cell Carcinoma of the Skin

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Homeostasis of stratified epithelia, such as the epidermis of the skin, is a sophisticated process that represents a tightly controlled balance between proliferation and differentiation. Alterations of this balance are associated with common human diseases including cancer. Here, we report the cloning of a novel cDNA sequence, from mouse back skin, that is induced by the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and codes for a hitherto unknown aspartic proteinase-like protein (Taps). Taps represents a potential AP-1 target gene because TPA-induced expression in epidermal keratinocytes critically depends on c-Fos, and co-treatment with dexamethasone, a potent inhibitor of AP-1-mediated gene regulation, resulted in impaired activation of Taps expression. Taps mRNA and protein are restricted to stratified epithelia in mouse embryos and adult tissues, implicating a crucial role for this aspartic proteinase-like gene in differentiation and homeostasis of multilayered epithelia. During chemically induced carcinogenesis, transient elevation of Taps mRNA and protein levels was detected in benign skin tumors. However, its expression is negatively associated with dedifferentiation and malignant progression in squamous cell carcinomas of the skin. Similar expression was observed in squamous skin tumors of patients, suggesting that detection of Taps levels represents a novel strategy to discriminate the progression state of squamous skin cancers. ***(Am J Pathol 2006, 168:1354–1364; DOI: 10.2353/ajpath.2006.050871)***

Stratified epithelia, such as epidermis and oral mucosa, provide a protective barrier for internal tissues against environmental stresses, chemical damage and bacterial infection.[1] The epidermis of the skin consists of a basal layer in which mitotically active cells are connected to the basement membrane separating the epidermis from the underlying dermis. The stem cells in the basal layer divide asymmetrically and give rise to daughter stem cells and transit amplifying cells. The latter divide a few more times and then withdraw from the cell cycle.[2] As they become localized to the suprabasal layers, the cells undergo a program of terminal differentiation involving the expression of various keratin proteins and finally leading to cornified cells lacking nuclei and other organelles.[1,3] During development, the embryo is first covered by a single-layered epithelium, the surface ectoderm. Maturation of the embryonic epidermis involves the formation of an intermediate layer between the basal layer and the periderm, a precursor of the stratum spinosum, followed by the development of stratum granulosum and stratum corneum at day E17.5. Subsequently, at day E18.5 the epidermis is fully developed, the periderm is shed, and each layer expresses a unique array of genes characteristic for its state of differentiation as in the adult epidermis.[3]

The switch between keratinocyte proliferation and differentiation in the adult epidermis is carefully controlled and compartmentalized into the basal and suprabasal cell layers, respectively. Several members of the serine and aspartic proteinase families have been shown to critically contribute to processes accompanying cornification.[4,5] Of these proteinases the aspartic proteinase

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cathepsin D has recently been described as necessary for the activation of transglutaminase 1 in concert with the regulation of cornified envelope proteins.[6] As the turnover of the epidermis culminates in the process of desquamation, in which individual corneocytes are shed from the outer stratum corneum, the proteolytic activity of cathepsin D is needed to degrade desmosomal protein complexes that hold together the outer squames in the differentiated suprabasal layers.[6–8] Because of the dynamic structure of the skin with proliferation in the basal layer and differentiation as well as shedding of the cells as anucleated squamae from the stratum corneum, a tight regulation of these processes is required to avoid pathological conditions such as hyperkeratosis, hyperplasia, or cancer.[3]

The current knowledge on processes involved in the development of epithelial cancer is primarily based on experiments using the well established chemically induced tumor model of mouse skin.[9–12] This multistage carcinogenesis model is based on an initiation step due to a single treatment of the mouse back skin with the mutagen 7,12-dimethylbenz[a]anthracene leading to irreversible DNA damage. Subsequent tumor promotion through repeated applications of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) results in the formation of multiple benign papillomas, some of which spontaneously progress to malignant, invasively growing squamous cell carcinomas (SCCs). Increased activity of the transcription factor AP-1 is required for tumor promoter-induced transformation of mouse keratinocytes and is implicated in the conversion of papillomas to carcinomas in the course of skin carcinogenesis.[11,13] AP-1 collectively describes a group of structurally and functionally related members of the Jun (c-Jun, JunB, and JunD), Fos (c-Fos, FosB, Fra-1, and Fra-2) and ATF (ATFa, ATF-2, and ATF-3) protein families that bind as homo- and heterodimers to TPA-responsive elements in the promoter regions of target genes.[14,15] AP-1 mediates gene regulation in response to a plethora of physiological and pathological stimuli and is implicated in various cellular processes, such as proliferation, differentiation, apoptosis, and transformation.[16,17] Resistance to chemically induced skin carcinogenesis observed in genetically modified mice confirmed that c-Fos and c-Jun critically contribute to transformation and malignant progression of epidermal keratinocytes.[18–22] Yet, functional AP-1 sites are also present in the regulatory regions of numerous genes correlated with keratinocyte differentiation indicating a pronounced function of AP-1 in the regulation of epidermal development and homeostasis.[23,24]

The multistage carcinogenesis model of mouse back skin is a valuable tool to study various stages of epithelial tumor development and to identify alterations in the genetic program that occur during the development of SCCs of the skin.[25,26] Recently, we used this model in combination with suppression subtractive hybridization to clone a cDNA library enriched for TPA-inducible genes in skin.[27–29] Here, we describe the cloning and characterization of a novel TPA-inducible aspartic proteinase-like gene with enhanced expression in premalignant skin tumors. Yet, expression is negatively correlated with malignant progression and disappears in undifferentiated squamous cell carcinomas of mouse and human. In concert with the restricted expression pattern in stratified epithelia of embryonic and adult mouse tissues, these data suggest a crucial role for this novel gene in the process of epithelial differentiation and its expression might be a valuable diagnostic tool for the discrimination of premalignant and malignant skin tumors, but also for other epithelial malignancies.

Materials and Methods

Animal Work and Sample Preparation

Animals were housed in specific pathogen-free and light-, temperature (21°C)-, and humidity (50 to 60% relative humidity)-controlled conditions. Food and water were available *ad libitum*. The procedures were in accordance with the principles and guidelines of the Arbeitsgemeinschaft der Tierschutzbeauftragten in Baden-Württemberg (ATBW) (officials for animal welfare) and were approved by the Regierungspräsidium Karlsruhe (AZ. 00/98 and AZ. 129/02). Dorsal skin of female NMRI and C57BL/6 ($fos^{-/-}$ and control littermates) mice was treated for the indicated time with 10 nmol of TPA, 50 μg of dexamethasone, or with acetone as control.[28] Skin tumors derived from female NMRI mice were generated according to the initiation-promotion protocol of chemically induced multistage carcinogenesis.[30]

Cell Culture

The mouse keratinocyte cell lines with variable tumorigenic potential *in vivo* have been previously described.[31–34] They were cultured at 34°C in a humidified atmosphere of 6% CO_2 and were split twice a week. Human HeLa cells were maintained in Dulbecco's modified Eagle's medium (Cambrex BioScience, Landen, Belgium) supplemented with 10% fetal calf serum (Sigma, St. Louis, MO) at 37°C in a humidified atmosphere of 6% CO_2.

Cloning of the Taps-Encoding cDNA and Expression Plasmids

A 1208-bp cDNA clone encoding mouse Taps was isolated from a cDNA library enriched for TPA-inducible genes in mouse back skin.[27] Another 302 nucleotides were cloned by the use of 5′-RACE technology following the manufacturer's recommendations (Invitrogen, Karlsruhe, Germany). To generate the eukaryotic pcDNA3.1-hTapsMyc/His plasmid, the ORF of human Taps was amplified using a human cDNA collection and on digestion with *Hind*III and *Not*I the polymerase chain reaction (PCR) product was cloned into pcDNA3.1(C)-Myc/His (Invitrogen). To introduce a D212N mutation within the active site, we performed PCR amplification with the primers 5′-TCC TGA AGG CCT TTG GGG TC-3′ and 5′-GAC CTG GGC CCC AGA ATT CAC C-3′. After digestion with *Stu*I and *Apa*I the PCR product was used to replace the corresponding sequence in a pBlue-

script SK$^-$ plasmid (Stratagene, La Jolla, CA) containing the Taps sequence. Finally, the Taps sequence with the D212N mutation was isolated on *Hind*III and *Xho*I digestion and cloned into a pcDNA3.1(C) plasmid (Invitrogen) to generate pcDNA3.1-DNhTapsMycHis. Positive clones of the 5′-RACE and all expression plasmids were sequenced using the ABI Prism BigDye Terminator cycle sequencing reaction kit and the ABI Prism 310 genetic analyzer (Applied Biosystems, Frankfurt, Germany) according to the manufacturer's instructions.

Transfection of HeLa Cells and Preparation of Protein Samples

HeLa cells were transfected with pcDNA3.1(C), pcDNA3.1-hTapsMyc/His, and pcDNA3.1-DNhTapsMyc/His using the GenePORTER reagent following the manufacturer's recommendations (Peqlab, Erlangen, Germany). Forty-eight hours after transfection, His-tagged Taps protein of cell extracts and supernatants was purified with Talon metal affinity resin according to the manufacturer's instructions (BD Bioscience, Heidelberg, Germany).

In Situ *Hybridization*

In situ hybridization was performed on 6-μm paraffin sections as described elsewhere.[27,35] The anti-sense riboprobe was synthesized from a linearized vector containing 426 nucleotides of Taps cDNA corresponding to nucleotides 533 to 959 of BC057938. As a control for specificity, sections were hybridized with the appropriate ^{35}S-labeled sense probe.

RNA Isolation, Northern Blot, and RQ-PCR Analysis

Isolation of total RNA from mouse back skin as well as chemically induced tumor samples and procedures for Northern blot analysis were described elsewhere.[26,28] Hybridization of Northern blots was performed with the ^{32}P-labeled cDNA probe cloned by the suppression subtractive hybridization screen (corresponding to 340 to 1563 bp of BC057938). The fragment was isolated by *Eco*RI digestion of the appropriate plasmid.[27] Hybridization with an 18S ribosomal RNA probe served as a control for equal loading and quality of total RNA. cDNA synthesis of total RNA and subsequent RQ-PCR analysis were performed as described previously.[28] The following primers were used for PCR detection: mTaps-for 5′-TTG GTG GCC TAA AGC AGT GTC-3′; mTaps-rev 5′-GCC AGT CTC CTC AA GCA TCT-3′. As an endogenous control levels of Hprt were used to standardize the amount of sample cDNA. All experiments were done in triplicates and data are given in means ± SEM.

Antibody Generation, Western Immunoblot, and Immunofluorescence Analysis

Two independent rabbit polyclonal antibodies raised against peptides corresponding to amino acids 222 to 234 or 317 to 328 of the predicted mouse Taps protein sequence (Supplemental Figure S1 at *http://ajp.amjpathol.org/*) were generated (Eurogentec, Seraing, Belgium). Antibody specificity was confirmed by Western immunoblot analysis with recombinant Taps-Myc/His protein that was purified from whole cell extracts of transfected HT1080 human fibrosarcoma cells (data not shown) and on peptide competition using the peptide corresponding to amino acids 222 to 234 (Supplemental Figure S1 at *http://ajp.amjpathol.org/*). Western immunoblot and immunofluorescence analysis were performed as described previously.[28] For immunofluorescence analysis polyclonal rabbit anti-Taps antibodies (whole serum after the third boost) were diluted 1:400 in phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin and 0.2% Tween 20. Anti-Taps antibody purified against the peptides used for generation was diluted 1:100 in 7.5% milk powder in PBS with 0.5% Tween 20 for Western immunoblot. Goat anti-rabbit-Cy3 (Dianova, Hamburg, Germany) and goat anti-rabbit-Alexa 488 (Invitrogen) were used as secondary antibodies for visualization and H33342 (Calbiochem, Schwalbach, Germany) for nuclear staining.

Human Skin Biopsies

Biopsy samples of patients with psoriasis, lichen ruber, eczema, and actinic keratoses were routinely taken for diagnostic purpose at the Department of Dermatology (University of Cologne). The skin lesions had not previously received any local treatment for at least 1 week. Skin biopsies from patients with keratoacanthoma or SCC were obtained from surgical excisions of the affected areas at the Department of Dermatology (University of Cologne). One part of the samples was immediately frozen in liquid nitrogen and stored at −80°C until used for histological examination. The patients signed the informed consent from the Department of Dermatology (University of Cologne), approved by the Institutional Commission of Ethics (AZ. 9645/96). Two experienced dermatologists confirmed the diagnoses independently.

Results

Identification and Cloning of a TPA-Inducible Aspartic Proteinase-Like Gene

Recently, we described the cloning of a cDNA library enriched for TPA-inducible genes expressed in mouse back skin.[26–29] Within this library, we discovered a 1.2-kb cDNA sharing a 3′ sequence of an ORF encoding a hitherto unknown protein. Applying 5′ RACE, we extended the knowledge on the cDNA sequence including an in-frame ATG at the beginning of a 1020 nucleotide

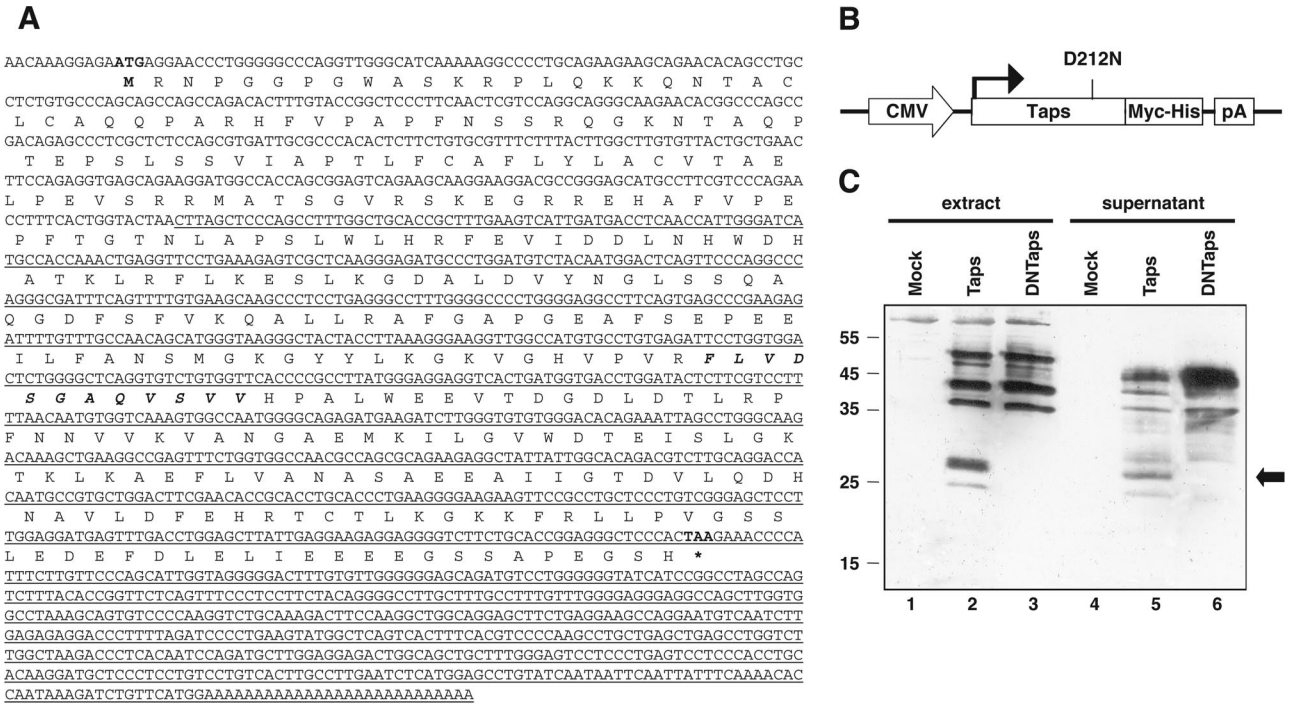

Figure 1. Taps cDNA encodes a novel aspartic proteinase-like protein. **A:** Sequence of mouse *Taps* cDNA including the ORF of 1020 nucleotides coding for a protein of 339 amino acids. The start and stop codon are indicated as **bold letters** and the motif typical for retroviral aspartic proteinases (amino acids 207 to 218) is shown in **bold italic letters**. **Underlined** is the sequence cloned by the suppression subtractive hybridization approach. **B:** Schematic drawing of the expression plasmid used for transient transfection of HeLa cells. The Taps ORF fused to a Myc-His Tag was cloned in an expression plasmid sharing the cytomegalovirus promoter (CMV) and a polyadenylation signal (pA). D212N indicates the introduction of a point mutation resulting in impaired proteolytic activity of Taps protein (DNTaps). **C:** HeLa cells were transfected with either the expression plasmid encoding the wild-type (Taps) or mutated protein (DNTaps), and the parental pcDNA3.1(C) vector as a negative control. Tagged Taps protein was enriched from cell extracts and supernatants of transfected cells by incubation with TALON resin and analyzed by Western immunoblot using the polyclonal anti-Taps antibody. The **arrow** highlights the product of autoproteolytic activity.

ORF encoding a protein of 339 amino acids (Figure 1A). Alignments using this cDNA sequence and the NCBI mouse genome database revealed the corresponding gene on mouse chromosome 6D1. Moreover, analysis with the GrailEXP online program (*http://compbio.ornl.gov/grailexp*) confirmed the presence of an intronless gene at this position (data not shown). Detailed inspection of the polypeptide sequence by motif search (*http://motif.genome.jp/*) revealed a region with significant homology to retroviral type aspartic proteinases (205 to 286 amino acids) including a DSG motif supporting the active site (207 to 218 amino acids). Therefore, we named this novel gene *taps* for TPA-inducible aspartic proteinase-like gene in *s*kin. Potential homologues of the mouse *taps* gene in other species were identified by blast search of the NCBI database including the hypothetical human protein FLJ25084 that shows 76% similarity on protein level (Supplemental Figure S1 at *http://ajp.amjpathol.org/*) and sequences in rat (XM_575593), cow (XM_580888), and chimpanzee (XM_525777).

To prove that *Taps* cDNA codes for a stable protein, we cloned its human ORF into an expression plasmid under the control of the CMV promoter (Figure 1B). The His-tagged Taps protein was purified from cell extracts and supernatant of transfected HeLa cells, respectively, and was studied by Western blot analysis with a newly generated polyclonal antibody raised against Taps peptides (Supplemental Figure S1 and S2 at *http://ajp.amjpathol.org/*). Several protein variants between 35 kd and 55 kd could be detected in cells transfected with the Taps expression plasmid, but not in the mock-transfected control (Figure 1C, lanes 1 and 2). In addition, we found expression of a smaller variant of ~28 kd, yet, this variant was no longer detected in cells transfected with an expression plasmid encoding Taps carrying a mutation in the DSG motif of the putative active site (DNTaps) implicating autoproteolytic activity of Taps (Figure 1C, lane 3). Finally, we could purify Taps protein from the supernatant of transfected cells demonstrating efficient secretion of Taps protein (Figure 1C, lanes 4 to 6). Similar data were also obtained in other cell lines and for the ORF of the mouse homologue (data not shown).

Taps Expression during Embryonic Development and in Adult Tissues

When we analyzed expression of Taps during normal mouse embryogenesis, *Taps* mRNA was detected by *in situ* hybridization in the epidermis of embryonic skin at day E16.5 (Figure 2, A and C), but was not observed at earlier time points (data not shown). At day E16.5, expression was also present in epithelia of the tongue and mucosa of the oral and nasal cavity (Figure 2, E and G, and data not shown). In adult tissues, we observed expression of *Taps* mRNA in single keratinocytes of the epidermis, in the cortex of the hair, and

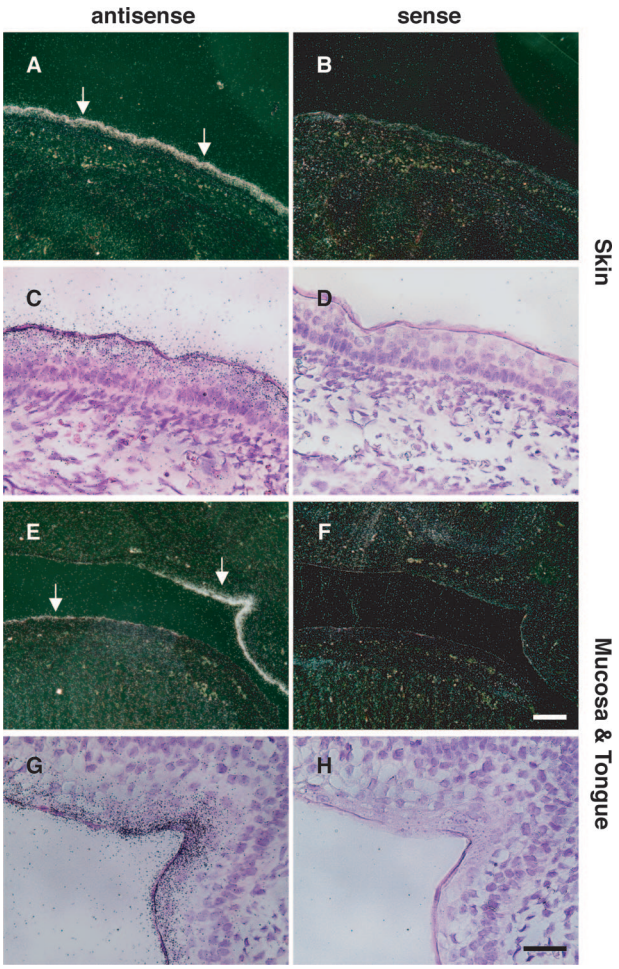

Figure 2. Expression of *Taps* mRNA in embryonic tissues. *In situ* hybridization with a ^{35}S-labeled anti-sense probe was performed on mouse embryonic sections on day E16.5 (**left**). The ^{35}S-labeled sense probe served as control for specificity of the signals (**right**). Images were counterstained with H&E and were done with dark-field (**A**, **B**, **E**, **F**) or bright-field (**C**, **D**, **G**, **H**) microscopy. Scale bars = 100 μm (dark field); 25 μm (bright field).

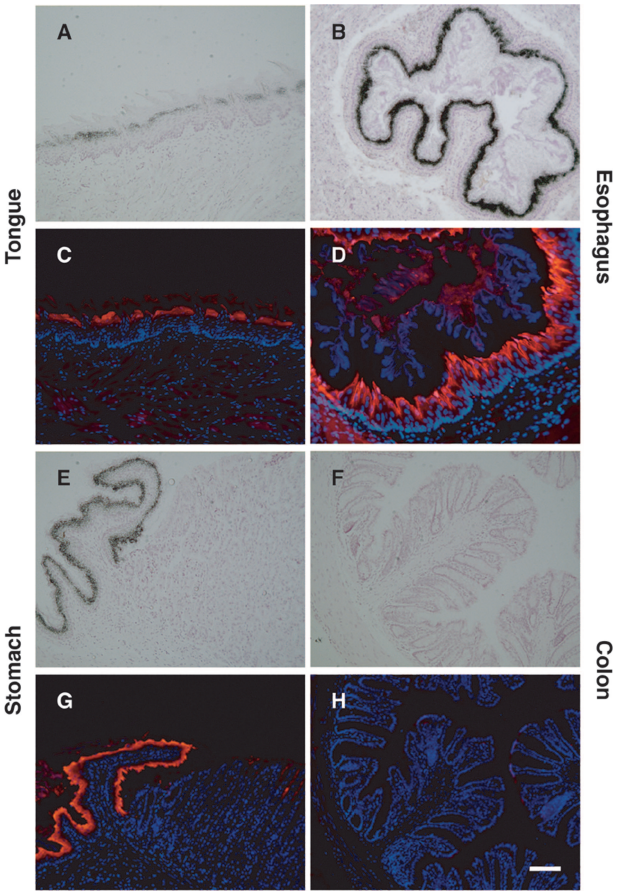

Figure 3. Expression of Taps mRNA and protein in adult mouse tissues. *In situ* hybridization with a ^{35}S-labeled anti-sense probe (black signal in **A**, **B**, **E**, and **F**) and IF analysis with an antibody specific for Taps in combination with a Cy3-labeled secondary antibody (red signal in **C**, **D**, **G**, and **H**) revealed Taps expression in stratified epithelia of tongue (**A**, **C**), esophagus (**B**, **D**), and forestomach (**E**, **G**). No expression was observed in simple epithelia such as colon (**F**, **H**). Sections were counterstained with H&E or H33342 (blue signal) for counterstaining of the nuclei. Images were taken with bright-field microscopy (**A**, **B**, **E**, and **F**) or immunofluorescence microscopy (**C**, **D**, **G**, and **H**). Scale bar = 100 μm.

restricted to superficial cells of other stratified epithelia, such as tongue, oral mucosa, esophagus, and forestomach (Figure 3, A, B, and E; and Supplemental Figure S3 at *http://ajp.amjpathol.org/*). An identical picture was observed when we determined the level of Taps protein using the newly generated rabbit polyclonal antibodies raised against Taps peptides (Figure 3, C, D, and G). In contrast, we did not observe Taps expression in simple epithelia of other tissues within the gastrointestinal tract, such as the glandular part of the stomach or the colon, suggesting that Taps is specifically involved in homeostasis and differentiation of stratified epithelia. Accordingly, we detected Taps expression only in suprabasal layers of all stratified epithelia that have been analyzed, which was confirmed, at least for the epidermis, by co-staining of newborn mouse skin sections with antibodies specific for keratin 10 and the late differentiation marker loricrin (Supplemental Figure S3 at *http://ajp.amjpathol.org/*).

Regulation of Taps Expression in TPA-Treated Mouse Back Skin

Having originally identified *taps* as a gene, whose expression is up-regulated upon short TPA treatment of mouse back skin, we then examined the kinetics of phorbol ester-mediated transcription. Mouse back skin was treated with TPA and at defined time points skin tissue was harvested for RNA preparation or was embedded in paraffin to generate skin sections. Northern blot analysis revealed elevated *Taps* mRNA levels even 1 hour after TPA application with a further increase up to 6 hours after TPA treatment (Figure 4A). Taps protein was specifically up-regulated in keratinocytes of the epidermis 6 hours after TPA application and persisted for 48 hours (Figure 4B). In accordance to these data, TPA-induced expression of *Taps* mRNA could be confirmed in an established mouse keratinocyte cell line with similar kinetics compared to skin (data not shown).

To elucidate the molecular mechanism involved in phorbol ester-induced Taps expression, we treated

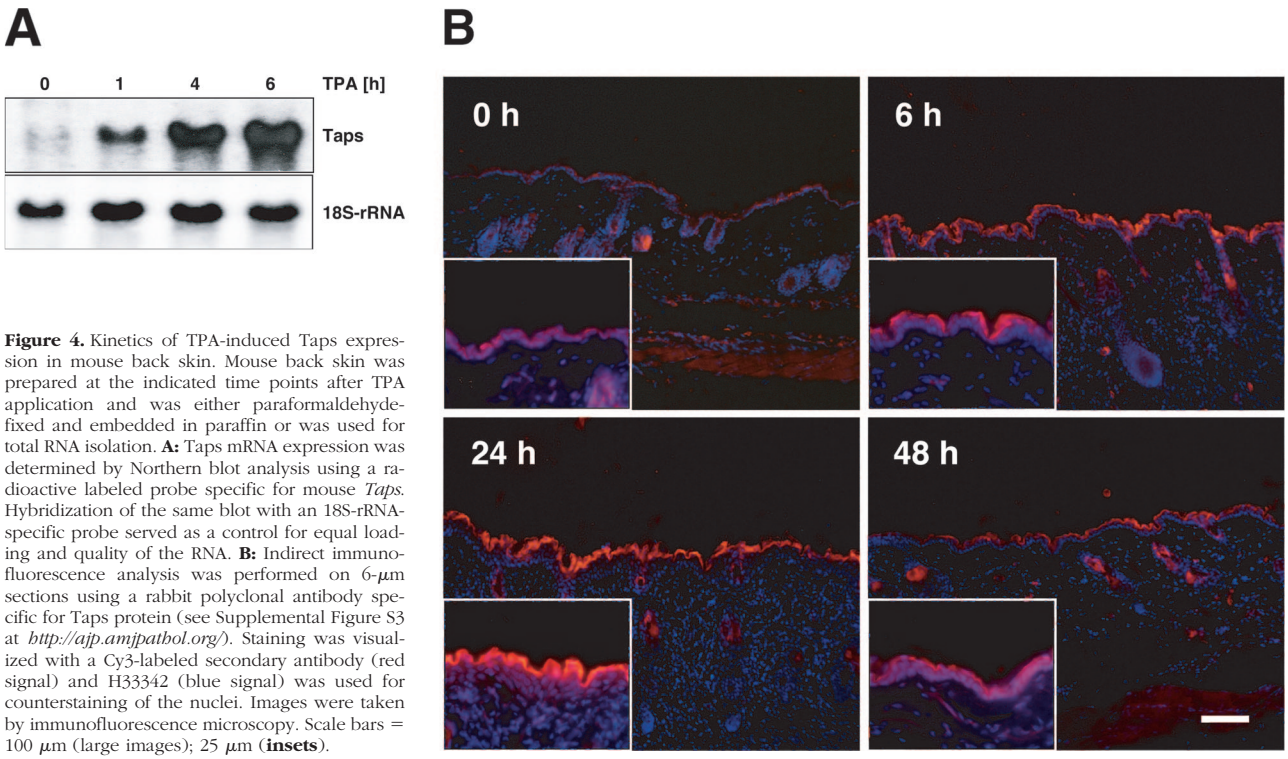

Figure 4. Kinetics of TPA-induced Taps expression in mouse back skin. Mouse back skin was prepared at the indicated time points after TPA application and was either paraformaldehyde-fixed and embedded in paraffin or was used for total RNA isolation. **A:** Taps mRNA expression was determined by Northern blot analysis using a radioactive labeled probe specific for mouse *Taps*. Hybridization of the same blot with an 18S-rRNA-specific probe served as a control for equal loading and quality of the RNA. **B:** Indirect immunofluorescence analysis was performed on 6-μm sections using a rabbit polyclonal antibody specific for Taps protein (see Supplemental Figure S3 at *http://ajp.amjpathol.org/*). Staining was visualized with a Cy3-labeled secondary antibody (red signal) and H33342 (blue signal) was used for counterstaining of the nuclei. Images were taken by immunofluorescence microscopy. Scale bars = 100 μm (large images); 25 μm (**insets**).

mouse back skin with TPA and dexamethasone (Dex). Dex is a synthetic glucocorticoid known to block TPA-induced hyperplasia and inflammation by interfering with the function of AP-1 and nuclear factor-κB.[36,37] Co-treatment of mouse back skin with Dex revealed an impaired induction of *Taps* mRNA (Figure 5A, top) suggesting a possible role of the AP-1 transcription factor in its phorbol ester-induced expression. Therefore, we studied *Taps* mRNA levels in mice deficient for c-Fos, a major member of AP-1.[16,17] Whereas basal expression was comparable between Fos-deficient and control littermates, we found no TPA-induced expression of *Taps* mRNA in mice lacking c-Fos (Figure 5A, bottom). These data demonstrate that c-Fos is indispensable for phorbol ester induction of Taps, which was further supported on protein level by immunofluorescence (IF) analysis (Figure 5B).

Altered Expression of Taps during Multistage Skin Carcinogenesis

Phorbol ester-induced and Fos-dependent expression in the epidermis argues for the possibility that Taps is also differentially expressed in the process of multistage skin carcinogenesis. Therefore, we performed quantitative RT-PCR with cDNA derived from control and TPA-treated back skin as well as 7,12-dimethylbenz[a]anthracene/TPA-induced papilloma and SCC samples. In line with the Northern blot analysis, strongly enhanced levels of *Taps* mRNA (20.7 $\pm$ 8.1-fold) were measured in TPA-treated back skin, and increased expression was also detected in papillomas (Figure 6A). Increased expression of Taps in papillomas was further confirmed on mRNA and protein level using tissue sections and *in situ* hybridization or IF analysis (Figure 6B). In contrast, no significant differences in total mRNA levels were found between SCC and control skin (Figure 6A). In agreement with the RQ-PCR data, SCC sections showed only minor Taps mRNA and protein expression that was restricted to keratinocytes surrounding the cornified inclusions within the tumor tissue, whereas no expression was observed in undifferentiated areas (Figure 6B). Thus, the expression pattern during chemically induced skin carcinogenesis suggested that total Taps levels are down-modulated during progression into undifferentiated and more aggressive tumor stages, which was further supported by its mRNA expression levels in established mouse keratinocyte cell lines (Figure 6C). In comparison to the keratinocyte cell line Reb, established from embryonic mouse skin, Taps expression was significantly increased in cells (SP1 and 308) that develop benign tumors after subcutaneous injection in nude mice. Highest Taps mRNA levels were observed for the cell lines PMK-R3 and 3P2 that both develop malignant tumors in nude mice but otherwise show an epithelial-like morphology under normal culture conditions (data not shown). Accordingly, significant levels of Taps protein were detected in the cell lines 308, PMK-R3, and 3P2 (Figure 6D). In contrast, Taps mRNA and protein expression were hardly detected in the cell lines PDV and CarB that are characterized by development of more aggressive tumors in nude mice in concert with spindle-shaped growth morphology under normal culture conditions (data not shown).

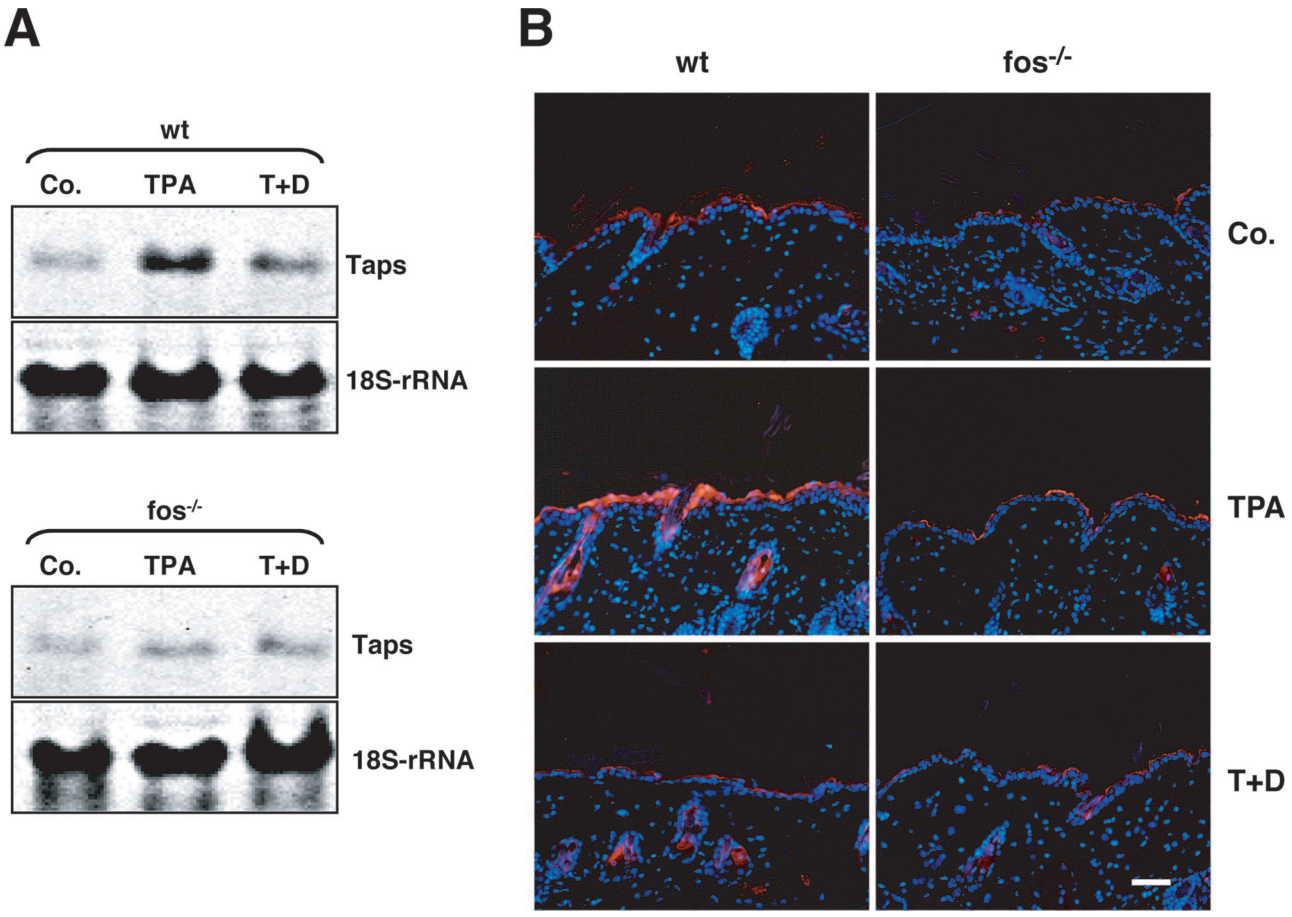

Figure 5. c-Fos is indispensable for TPA-induced Taps expression. Back skin of wild-type (wt) and fos-deficient mice (*fos*$^{-/-}$) was treated with acetone (Co.), TPA, or TPA and dexamethasone (T+D). Animals were sacrificed 6 hours after treatment and skin samples were used for total RNA isolation and Northern blot analysis as described in Figure 4A (**A**) or were used for skin sections and IF analysis as described in Figure 4B. Images were taken by immunofluorescence microscopy. Scale bar = 50 μm.

Expression of Taps in Human SCC and Nonneoplastic Lesions

To investigate, whether Taps expression correlates with human skin carcinogenesis, we performed IF analysis on tissue sections of benign keratoacanthoma and malignant SCC. We found Taps protein expressed in keratinocytes of the stratum granulosum within normal skin adjacent to the tumor (Figure 7A) and enhanced expression in cells surrounding the cornified inclusions within the keratoacanthoma (Figure 7B). According to the situation in the chemically induced mouse tumors, we could detect Taps expression in differentiated areas of SCC tumors (Figure 7C), but not in undifferentiated tumors (Figure 7D) suggesting that Taps expression is associated with aberrant differentiation rather than tumor progression. To provide further evidence for this hypothesis, we studied Taps protein expression on tissue sections of patients with various nonneoplastic skin lesions including psoriasis, lichen ruber, as well as eczema and actinic keratosis as an analogue of benign papilloma. In all analyzed lesions, we detected high Taps protein levels restricted to keratinocytes of the stratum granulosum and in psoriasis a further signal in the stratum corneum (Figure 8).

Discussion

In the present study, we describe the cloning and characterization of a novel TPA-inducible cDNA expressed in keratinocytes of mouse back skin that codes for a hitherto unknown protein that we called Taps. Taps mRNA and protein are restricted to stratified epithelia in embryos and adult mice and its expression is transiently elevated during skin carcinogenesis.

Several findings argue for an involvement of AP-1, a well known downstream target of TPA, in the regulation of *taps* gene expression. TPA-mediated transcriptional induction of *Taps* is repressed by dexamethasone, a synthetic glucocorticoid, which is best explained by negative interference between the activated glucocorticoid receptor and AP-1.[36,37] The involvement of AP-1 for induced Taps expression *in vivo* was confirmed by an impaired expression in TPA-treated back skin of mice lacking c-Fos, which show remarkable abnormalities in chemically induced skin carcinogenesis.[19] In line with these data, we found a putative AP-1 binding motif upstream of the postulated transcriptional start site conserved in mouse and human (Rhiemeier V, Hess J, and Angel P, unpublished data).

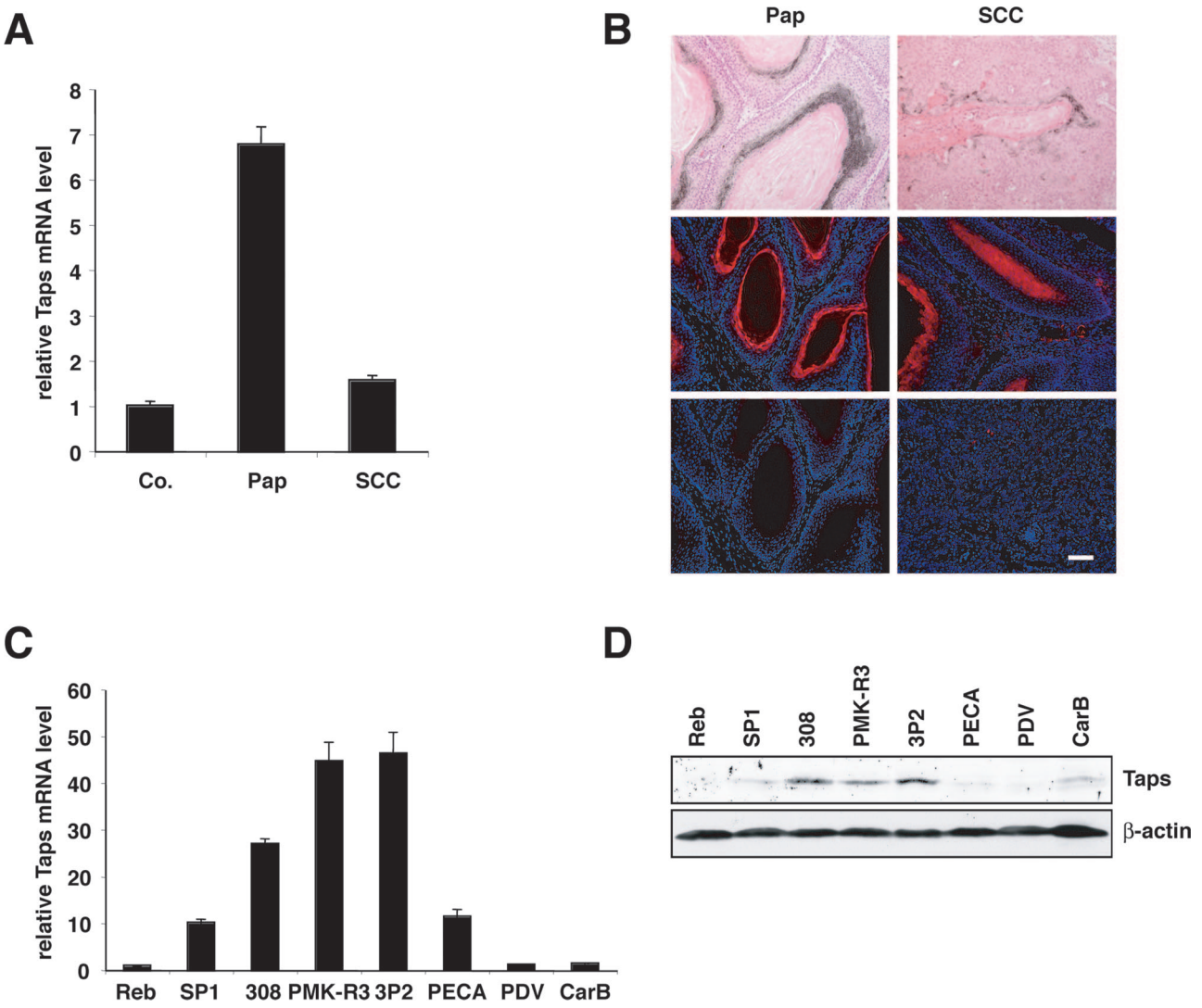

Figure 6. Expression of Taps during multistage skin carcinogenesis. Mouse back skin (Co.), benign (Pap), and malignant (SCC) tumor samples derived from the protocol of chemically induced carcinogenesis were used for total RNA isolation and cDNA synthesis. **A:** Quantitative RT-PCR was performed with *Hprt* as internal control and relative expression of control skin was set to one. Bars represent mean values ± SEM of an experiment that was done in triplicate. **B:** *In situ* hybridization with a ^{35}S-labeled anti-sense probe (black signal, **top**) and IF analysis with an antibody specific for Taps on papilloma and a well differentiated SCC (red signal, **middle**) as well as on a dedifferentiated malignant tumor (**bottom right**). As a control a benign tumor was incubated with the second antibody only (**bottom left**). Sections were counterstained with H&E or with H33342 (blue signal) for counterstaining of the nuclei. The ^{35}S-labeled sense probe served as a control for specificity of signals (data not shown). Images were taken by bright-field or immunofluorescence microscopy. **C** and **D:** Total RNA and cell extracts were prepared from several mouse keratinocyte cell lines characterized by their potential to form benign or malignant tumors after subcutaneous injection in nude mice. RQ-PCR was performed as described in **A** and bars represent mean values ± SEM of an experiment that was done in triplicate. Western immunoblot using the rabbit polyclonal anti-Taps antibody was done to demonstrate protein expression in these cell lines. Incubation of the same blot with the polyclonal anti-β-actin antibody served as a control for quantity and quality of the protein extracts. Scale bar = 100 μm.

Phorbol esters, such as TPA, are the most potent and most frequently used tumor promoters in mouse models of skin carcinogenesis. Skin carcinogenesis is a multistage disease characterized by differential expression of genes that are critical mediators of phenotypic and physiological alterations in cells on their way to malignancy.[10,11] As observed in most other malignancies, there are several characteristic precursor lesions known for skin cancer. Therefore, identification and characterization of genes with altered expression at early stages of tumor development are the basis for a comprehensive understanding of the molecular mechanisms and will be useful to elucidate new targets for innovative diagnostic and therapeutic strategies of human cancer. Recent studies applying global gene expression analysis on samples derived from the chemically induced mouse model of skin carcinogenesis demonstrated elevated expression of TPA-inducible genes also in benign and malignant skin tumors.[25–29] Although we found elevated Taps levels in benign mouse tumors, its expression is restricted to keratinocytes surrounding the cornified inclusions that are characteristic for a deregulated differentiation in premalignant tumors. Accordingly, we detected Taps mRNA and protein only in differentiated SCCs with cornified inclusions, but not in undifferentiated areas of malignant tumors implicating that elevated Taps expression is associated with aberrant differentiation rather than transformation of keratinocytes. This hypoth-

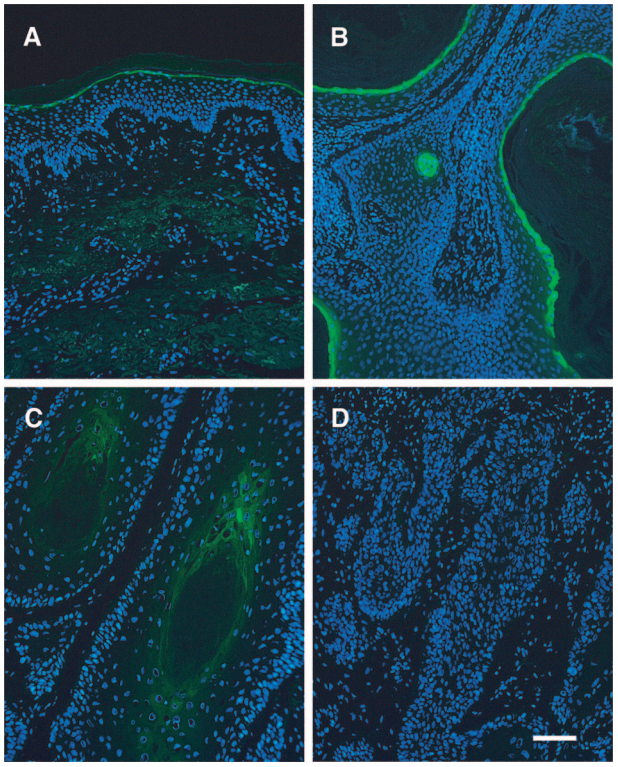

Figure 7. Expression of Taps protein in human skin tumors. Indirect IF analysis with the polyclonal anti-Taps antibody revealed Taps expression in the stratum granulosum of normal skin adjacent to the tumor tissue (**A**), in a keratoacanthoma (**B**), and in a SCC with cornified inclusions (**C**), but not in a dedifferentiated SCC (**D**). Staining was visualized with an Alexa 488-labeled secondary antibody (green signal) and H33342 (blue signal) was used for counterstaining of the nuclei. Images were taken by immunofluorescence microscopy. Scale bar = 100 μm.

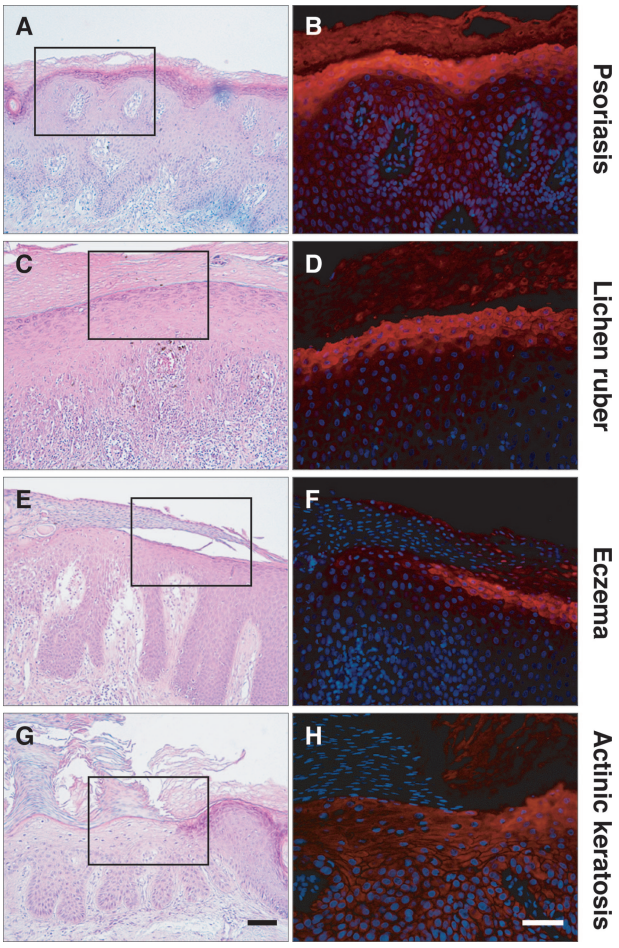

Figure 8. Expression of Taps protein in human skin lesions. Indirect IF analysis with the polyclonal anti-Taps antibody revealed expression of Taps protein (red signal) in the stratum granulosum in various skin lesions like psoriasis (**B**), lichen ruber (**D**), eczema (**F**), and in actinic keratosis (**H**). Nuclei were counterstained with H33342 (blue signal). IF analysis was performed as described in Figure 4B and the corresponding H&E-stained images (**A**, **C**, **E**, **G**) demonstrate lower magnification of the respective immunofluorescence images. The **black rectangles** within the images depict the area shown in the immunofluorescence analysis. Scale bars = 100 μm (H&E); 50 μm (IF).

esis was further supported by the lack of *Taps* mRNA in established keratinocyte cell lines characterized by aggressive tumor growth after subcutaneous injection into nude mice and spindle cell morphology in culture. Moreover, we detected high levels of Taps protein in tissue sections of patients with nonneoplastic skin lesions with varying degrees of hyperkeratosis as well as in samples of actinic keratosis, a precancerous lesion that might progress into SCC, which displays hyperkeratosis and atypical keratinocytes predominantly within the basal cell layer. Although psoriasis and eczema are characterized by acanthotic epidermis, papillomatosis, and parakeratosis, a thickened stratum granulosum and basal degeneration are typical in lichen ruber. Thus, strong expression of Taps in the keratinocytes of the stratum granulosum of all types of lesions further corroborated the hypothesis that Taps expression is correlated with keratinocyte differentiation and might be critical for the homeostasis of the epidermis. Clearly, more detailed and comprehensive studies on Taps expression in premalignant and malignant human tumors will be required to determine, whether Taps detection represents a novel strategy to discriminate the progression state of squamous cell cancers of the skin, but also of other epithelial malignancies.

The amino acid sequences of Taps proteins from several species exhibit structural properties relating them to the aspartic proteinase superfamily. However, whereas most cellular aspartic proteinases known today are two-domain proteins with one Asp residue in each domain contributing to the active site,[38] we found only one motif within the amino acid sequence of Taps. Thus, Taps appears to be related to the retroviral members rather than to the known eukaryotic members of this class. Recently, GCDFP-15/pg17 was shown to be another example of a eukaryotic protein being related to retroviral aspartic proteinases.[39] GCDFP-15/pg17 is secreted by various exocrine glands including the seminal vesicles, salivary, as well as sweat glands, and is expressed in breast cancer cells.[40] It is worthwhile to mention that although GCDFP-15/pg17 expression is a generally accepted characteristic of apocrine carcinomas, its expression seems to be transient and restricted to low-grade breast cancer with the percentage of GCDFP-15/pg17-positive cells negatively correlated with primary tumor size in infiltrating carcinomas.[41,42] Thus, the expression

profile of Taps and GCDFP-15/pg17 suggests a common suppressive function for distinct aspartic proteinases during epithelial malignancy, specifically in the process of tumor invasion and metastasis. We could not detect any obvious sequence similarity, yet both proteins exhibit some common functional properties. It has been demonstrated that GCDFP-15/pg17 exists as a dimer and a tetramer of glycosylated 17-kd subunits in various body fluids and shows proteolytic activity for fibronectin *in vitro*.[39,43] Similarly, we have experimental evidence that Taps is part of a dimeric and/or multimeric complex and that the purified protein is active on fibronectin, although with low efficiency (V. Rhiemeier, J. Hess, and P. Angel, unpublished data). Most recently, Bernard and colleagues[44] published the identification of the skin aspartic protease (SASPase) that is identical to human Taps, and demonstrated degradation of insulin and to a lesser extent casein by recombinant SASPase derived from bacteria in a highly sensitive, fluorescence-based proteinase assay. Moreover, processing by autoactivation was inhibited by D212A or D212E mutation and by Indinavir, a potent HIV protease inhibitor used in AIDS therapy. In line with these data, we detected autoproteolysis in transfected HeLa cells for which a functional DSG motif is absolutely essential. Altogether, these experimental data clearly demonstrate that Taps represents a novel functional aspartic proteinase. Although *taps* represents an intronless gene, we obtained a complex pattern of three protein variants between 35 and 55 kd in transfected cells, suggesting posttranslational modifications and/or alternative start codon usage for translation. Indeed, we found a second ATG in the ORF of *Taps* mRNA that is in-frame and conserved in all species. Preliminary experiments demonstrate that expression of the 35-kd variant critically depends on the presence of the second ATG suggesting that alternative usage of translational start sites contributes, at least in part, to the observed complexity of protein variants in transfected cells (Supplemental Figure S1 at *http://ajp.amjpathol.org/*). It is worthwhile to mention that all variants share the conserved domain for aspartic proteinases and are able to form dimers *in vitro* (data not shown).

The enhanced expression of Taps in aberrantly differentiating keratinocytes of nonneoplastic skin lesions and skin tumors together with its restricted expression pattern in stratified epithelia during embryogenesis and in adult tissues argues for a crucial function in terminal differentiation of epithelial cells. An important role for aspartic proteinases in the process of epithelial differentiation has been well documented for cathepsin D, which is involved in the control of cell differentiation during normal development. Increased expression levels and function of cathepsin D were found in the skin depending on the stage of epidermal differentiation.[8] Moreover, cathepsin D is functionally linked to transglutaminase 1 activity, protein expression of cornified envelope proteins during epidermal differentiation, and desquamation of the stratum corneum by proteolytic cleavage of desmosomal proteins.[6] In the future, the establishment of genetically modified mouse models in which Taps expression is altered in epithelial tissues will be required to address its *in vivo* function in stratified epithelia and to determine whether lack of Taps expression supports carcinogenesis due to altered differentiation.

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