Identification and Characterization of Novel Genes Modulated in the Thyroid of Dogs Treated with Methimazole and Propylthiouracil*

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Induction of cell proliferation by mitogen or growth factor stimulation leads to the specific stimulation or repression of a large number of genes. To better understand differentiated epithelial cell growth regulation, we have initiated a study to identify genes which are regulated by the thyrotropin-dependent mitogenic pathway in dog thyroid cells. A thyroid cDNA library was prepared from a methimazole and propylthiouracil-treated dog and differentially screened with probes derived from control or stimulated thyroids. Among 19 clones isolated, 6 encode known proteins (inwardly rectifying potassium channel, nucleosome assembly protein, ribosomal protein L7, elongation factor 1α, non-muscle myosin light chain, and heat shock protein 90β). The 13 others correspond to proteins whose function is unknown. Among them, 5 correspond to mRNAs whose expression was modulated by mitogenic stimulation of thyrocytes in primary culture. A preliminary characterization of two of these cDNAs is reported: clone 5, which might represent a novel, atypical protein kinase, and clone 3, which contains ankyrin-like repeats, suggesting that it might interact with other proteins.

Induction of cell proliferation by mitogen or growth factor stimulation leads to the specific and sequential expression of a large number of genes (1–8): immediate early genes, induced by mitogenic stimulation leads to the specific and sequential expression of a large number of genes. To better understand differentiated epithelial cell growth regulation, we have initiated a study to identify genes which are regulated by the thyrotropin-dependent mitogenic pathway in dog thyroid cells. A thyroid cDNA library was prepared from a methimazole and propylthiouracil-treated dog and differentially screened with probes derived from control or stimulated thyroids. Among 19 clones isolated, 6 encode known proteins (inwardly rectifying potassium channel, nucleosome assembly protein, ribosomal protein L7, elongation factor 1α, non-muscle myosin light chain, and heat shock protein 90β). The 13 others correspond to proteins whose function is unknown. Among them, 5 correspond to mRNAs whose expression was modulated by mitogenic stimulation of thyrocytes in primary culture. A preliminary characterization of two of these cDNAs is reported: clone 5, which might represent a novel, atypical protein kinase, and clone 3, which contains ankyrin-like repeats, suggesting that it might interact with other proteins.

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EXPERIMENTAL PROCEDURES

Animal tissues used for this work are the same as those of a previous study conducted in our laboratory (19). The dogs used in this study were used for cardiological studies, and their thyroids were resected before these experiments. Briefly, dogs were treated by oral administration for 4 weeks of methimazole (MM) (2 × 60 mg/day strumazol) and propylthiouracil (PTU) (2 × 150 mg/day propylthiourit) in order to increase the circulating TSH level. Triiodothyronine and thyroxine concentrations in the serum were followed by radioimmunoassay to ensure that the treatment had been effective. On the day of the experiment, 1 h prior to thyroid resection, the dogs received 50 mg/kg bromodeoxyuridine by intravenous injection. Bromodeoxyuridine labeling analyses were performed as described (20). Tissues for PCNA immunohistochemistry and RNA preparation were obtained from MM/PTU treated and untreated dogs. The immunohistochemical procedure for PCNA immunohistochemistry was used as described previously (21). The method involves methanol fixation of the tissue (previously snap-frozen in this case), followed by embedding in paraffin.

Dog thyroid follicles were obtained as detailed previously (22). The follicles were seeded in 100-mm Petri dishes and cultured in a control medium consisting of Dulbecco’s minimal essential medium (Life Tech-
nologies, Inc.), Ham's F-12 (Life Technologies, Inc.), MCDB 104 (Life Technologies, Inc.), ZAP-cDNA synthesis and cloning system of Stratagene, according to the manufacturer's protocol. The resultant library contained approximately 10^6 recombinant phages. The library was amplified once by the plate lysate method to obtain 2.4 × 10^10 plaque-forming units/ml. To assess size distribution of inserts in the cDNA library, individual plaques were randomly selected and plate lysate stocks were generated by infection of XL-1 blue Escherichia coli cells. Phage DNA was extracted and restricted by EcoRI/XhoI, and this was then electrophoresed on 1% agarose gels to visualize insert sizes. The mean size of the randomly selected cloned inserts was 2.6 kb. Poly(A)^+ RNA from in vivo quiescent or stimulated thyroid tissues was used as template for the synthesis of single-stranded digoxigenin-labeled cDNA probes using hexamers and Moloney murine leukemia virus reverse transcriptase following the protocol suggested by the manufacturer (Dog Labeling kit, Boehringer Mannheim). For differential screening, the library was plated at low density (approximately 6000 plaque-forming units/13-cm diameter dish), and duplicate nylon membranes (Qiabrine, Qiagen) lifts were made from each dish. Filters were washed 4 times for 10 min in 2× SSC, 0.1% SDS at room temperature and 4 times for 20 min in 0.1× SSC, 0.1% SDS at 60°C. They were then autoradiographed at −70°C using Hyperfilm MP (Amersham Corp.) and Siemens intensifying screens.

RESULTS
Construction of a cDNA Library from a Thyroid of a MM/PTU-treated Dog—To construct a TSH-stimulated thyroid cDNA library, a dog was treated with methimazole and propylthiouracil as described (see above). At the time of thyroid resection, the dog was biochemically hypothyroid and its thyroid was thus chronically stimulated by TSH. Serum thyroxine and triiodothyronine levels were 0.5 μg/dl and <30 ng/dl, respectively, versus 1.1 ± 0.1 μg/dl and 37 ± 6 ng/dl in control dogs. Histological examination showed markedly a hyperplastic thyroid with almost total absence of colloid in the antithyroid drug-treated animal, and normal follicles containing colloid in the control thyroids. Bromodeoxyuridine labeling analysis demonstrated nuclear labeling in 8.1% of the thyroid cells of the treated dog, whereas the thyroid cells of control animals showed only 0.17 ± 0.07% labeled nuclei (mean ± S.E.).

Total RNA was extracted from the thyroid of the MM/PTU-treated dog and the poly(A)^+ RNA fraction was isolated. The quality of the poly(A)^+ RNA was confirmed by Northern blot analysis, using a thyroglobulin probe, which detected an intact 8.5-kb mRNA (data not shown). This poly(A)^+ RNA was used as template for the construction of an oligo(dT)-primed cDNA library in the λZAP II phage vector. Identification of Modulated Genes by Differential Screening—The screening of 250,000 plaques, with cDNA probes from control and stimulated thyroids, yielded 19 plaques which, after a second screening, showed consistent differential hybridization. Among these, 11 showed overexpression in the treated thyroid, and 8 showed underexpression. All cDNA inserts were partially sequenced and the sequences were compared to the GenBank and EMBL data bases, using the BLAST program for nucleic acids and proteins (Table I). Six clones were identified as previously characterized genes. These known clones coded for a inwardly rectifying potassium channel (IRK-2), some assembly protein-1 (NAP-1), ribosomal protein L7, elongation factor 1a (EF-1a), non-muscle myosin light chain (MLC), and heat-shock protein-90 β (hsp90). Four other clones (4, 5, 137, and 169) showed significant similarities to DNA sequences in the data bases but corresponded to proteins whose functions are unknown (accession numbers: 4, >gb/D14812/HUMORF16; 5, >gb/Z43809/H5C1L6E21; 137, >emb/Z43809/H5C1L6E21; 169, >gb/M85912/M85912). The remaining 9 clones did not match any known gene in data bases.

In Vivo mRNA Regulation—Total RNA was extracted from the thyroids of several control or MM/PTU-treated dogs and subjected to Northern blot analysis. Acridine orange staining,
rather than β-actin, glyceraldehyde-3-phosphate dehydrogenase, or cyclophilin hybridization, was used to normalize the amount of total RNA transferred onto the filter. Indeed, these three genes are modulated in our system (data not shown). One representative experiment is illustrated in Fig. 1. Clone 3 mRNA is up-regulated in the stimulated dog thyroids. Clone 165 mRNA level was highly variable between dogs, enhanced in some animals or attenuated in others. It was therefore not further characterized. Clones 5, 4, 2, 45, 143 (hsp90), and 134 (NAP) transcript levels were all reduced in stimulated thyroids. No modulation was observed for clone 44 (EF-1α) and clone 169, and no signal was detected for clones 101, 51, and 53 (IRK-2). Hybridization with clones 16, 59, 137, and 166 revealed only a smear, but no discrete transcript. All these data were confirmed in several dogs. Thus, these Northern studies demonstrate the modulation of five unknown genes in stimulated thyroids.

**Analysis of Tissue Distribution**—The following dog tissues were harvested shortly after sacrifice, snap-frozen in liquid nitrogen, and investigated by Northern blotting: thyroid, spleen, liver, lung, heart, skeletal muscle, kidney, adrenal, ovary, testis, stomach, pancreas, large intestine, lymph node, cerebrum, and cerebellum. Clones 2, 3, 4 (Fig. 2), 59, 165, and 169 (Table II) showed no particular tissue distribution pattern. They were detected in most of the tissues analyzed, with a particularly important expression in the adrenal for clone 3, and in the testis, thyroid, and brain for clone 4 (Fig. 2). Clone 5 showed strong thyroid specificity although it could also be clearly seen in both the ovary and cerebrum (Fig. 2). Clones 45 and 51 showed a much higher expression in the thyroid than in the other tissues. Clone 53 (IRK-2) was present only in skeletal muscle, cerebrum, and cerebellum, which was not unexpected (Table II). Clone 101, interestingly, appeared to be confined solely to the liver. To obtain qualitative estimates of the proliferative state of the tissues, those were stained with a mono-

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**TABLE I**

Identification of 19 differentially expressed cDNAs
Sequences of partial cDNA clones were compared with EMBL and GenBank data bases. (?, unknown gene)

| Clone | Insert size (kb) | Identification |
|-------|-----------------|----------------|
| Overexpressed | | |
| 3 | 3 | ? |
| 44 | 1.8 | EF-1α |
| 51 | 0.7 | ? |
| 53 | 1.9 | IRK-2 |
| 59 | 1.3 | ? |
| 101 | 2.8 | ? |
| 137 | 0.8 | ? |
| 143 | 2.5 | Ribosomal protein L7 |
| 164 | 0.7 | MLC |
| 165 | 0.6 | ? |
| 167 | 2 | ? |
| 169 | 1.4 | ? |
| Underexpressed | | |
| 2 | 1.2 | ? |
| 4 | 0.8 | ? |
| 5 | 1 | ? |
| 16 | 3.2 | ? |
| 45 | 2.2 | ? |
| 134 | 2.5 | NAP |
| 143 | 1.5 | Hsp 90 |
| 166 | 4.2 | ? |

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**TABLE II**

Summary of RNA tissue distribution of 5 clones
Total RNA was extracted from dog tissues and subjected to Northern blot analysis (−, no visible expression; +, barely to moderate expression; +++, moderate to strong expression). For PCNA immunohistochemistry, the proliferative activity was reported using the following ascending scale: −, +, ++, ++++, where − represents no positive nuclei and ++++ represents the positive control level (mouse small intestine).

| Clone number | Thyroid | Spleen | Liver | Lung | Heart | Skeletal muscle | Kidney | Adrenal | Ovary | Testis | Stomach | Pancreas | Large intestine | Lymph node | Cerebrum | Cerebellum | PCNA labeling |
|--------------|---------|--------|-------|------|-------|-----------------|--------|---------|-------|--------|---------|---------|-----------------|-----------|---------|----------|---------------|
| 53 | + | + | + | + | + | ++ | + | + | ++ | + | ++ | + | + | + | + | + |
| 59 | + | + | + | + | + | ++ | + | + | ++ | + | ++ | + | + | + | + | + |
| 101 | + | + | + | + | + | ++ | + | + | ++ | + | ++ | + | + | + | + | + |
| 165 | + | + | + | + | + | ++ | + | + | ++ | + | ++ | + | + | + | + | + |
| 169 | + | + | + | + | + | ++ | + | + | ++ | + | ++ | + | + | + | + | + |

**FIG. 1.** *In vivo* mRNA levels of 8 different clones. The animals were treated with MM and PTU as described under “Experimental Procedures.” C, untreated; MM/PTU, treated. Total RNA was extracted and subjected to Northern blot analysis. The right part of the figure shows acridine orange staining of the gel.

**FIG. 2.** RNA tissue distribution of 6 clones and PCNA immunohistochemistry of dog tissues. Total RNA was extracted from dog tissues and subjected to Northern blot analysis. For PCNA immunohistochemistry, the proliferative activity was reported using the following ascending scale: −, +, ++, ++++, where − represents no positive nuclei and ++++ represents the positive control level (mouse small intestine).
clonal antibody (PC10) against proliferating cell nuclear antigen (PCNA). The number of PCNA positive nuclei in a tissue is supposed to be closely correlated to that tissue’s growth fraction (21). However, the proliferative activity, qualitatively assessed by two observers, suggests no correlation between the tissue mRNA expression level and the level of proliferation (Fig. 2, Table II).

**In Vitro mRNA Regulation**—In primary cultures of dog thyroid cells, the thyrocytes were cultured for 4 days without mitogenic agents to allow cells to spread and reach a quiescent state. In the absence of stimulating agents, virtually no labeling of nuclei was observed during the 48 h after the addition of [3H]thymidine to the culture medium. Addition of TSH (1 milliunit/ml), forskolin (10⁻⁵ M), an adenylate cyclase activator, EGF (25 ng/ml), and FCS (10%) at day 4 induced, after a lag of about 18 h, a progressive increase in the fraction of [3H]thymidine incorporating nuclei (30). The results of Northern blot hybridization analyses are presented Fig. 3, 4, 5, 6, and in Table III.

The clone 5 mRNA levels peaked after 20 h incubation in the presence of TSH and decreased after 48 h (Fig. 3). EGF also increased clone 5 mRNA levels after 6–24 h (Table III). Chronic stimulation by EGF and FCS did not change the expression of this clone (Fig. 3).

Clone 3 mRNA was down-regulated by forskolin (Fig. 4A) and TSH (not shown) for the first 24 h. It was increased thereafter, but never exceeded the control levels, even after a 7 day stimulation (not shown). EGF led to an up-regulation, with acute (Fig. 4A) as well as with chronic stimulation (Table III). The mRNA content was already enhanced after 8 h of EGF stimulation, and increased further with longer incubation times. The negative effect of forskolin or TSH on mRNA levels was also observed on the EGF stimulation, since the simultaneous addition of EGF and TSH for 48 h led to a lower mRNA level than when EGF was added alone (Fig. 4B). The effects of EGF and EGF + TSH on clone 3 mRNA content were reproduced by HGF (50 ng/ml), another potent mitogenic agent for thyrocytes (31), and HGF + TSH. However, the increase observed with HGF was less pronounced, and the TSH inhibition was smaller (Fig. 4B).

Clone 2 mRNA levels were slightly increased after TSH treatment whereas a 3-day EGF + FCS treatment led to a clear accumulation (Fig. 5, Table III). Acute stimulation by EGF also enhanced clone 2 mRNA levels (Table III).

The mRNA levels of two identified clones (hsp90 and NAP) were also studied (Fig. 6). Treatment with forskolin led first to a decrease of hsp90 and NAP-1 mRNA levels, then after 20 h to an increase. Hsp90 mRNA control levels increased in parallel with the age of the culture; they were further enhanced by chronic treatment with EGF (Table III).

Analysis of the data obtained for the other clones presented in Table III shows that clone 4 mRNA expression was enhanced by TSH and EGF, and clone 45 mRNA amounts were increased by TSH, but remained equal after EGF treatment. As in vivo, no modulation was observed for EGF and clone 169, no signal was detected for clones 101, 51, and IRK-2, and hybridization with clones 16, 59, 137, and 166 was uninterpretable.

**Characterization of Clones 5 and 3**—Four clones were selected for further characterization: clones 5 and 3 (based on their modulated mRNA levels), clones 45 and 51 (based on their tissue distribution). The complete sequences of clones 5 and 3 were determined. Hydrophobicity profile of the predicted 343-amino acid sequence of clone 5 according to the analysis of Kyte and Doolittle (48) revealed the presence of a stretch of 20 hydrophobic amino acids, which might represent a potential transmembrane region. Protein sequence comparison of clone 5 with the data bases showed ~30% identity with the protein kinase family (serine-threonine kinases). However, among the 12 well conserved subdomains of the kinase catalytic domain (32), only two are present in our clone: subdomains VIII and IX, with an homology of ~80% (Fig. 7a). Clone 3 encodes a 659-amino acid protein containing 6 ankyrin-like repeats (Fig. 7b). The DNA library accession numbers of clones 3 and 5 are, X99145 and X99144, respectively.

**DISCUSSION**

To identify new genes modulated by the TSH-dependent mitogenic pathway, we performed a differential screening of a thyroid cDNA library from a dog treated in vivo with MM/PTU. The use of an in vivo chronically stimulated thyroid insured, with no a priori assumption, that the real in vivo growth pathway was investigated. This led to the identification of 11 clones showing overexpression and 8 showing underexpression. While 6 clones encode known proteins, the 13 others have not been described to date. Among them, 4 (5, 4, 137, and 169) showed significant similarities to DNA sequences in the data bases but corresponded to proteins whose function is unknown.

**TABLE III**

**Summary of in vitro mRNA regulation studies**

Dog thyrocytes in primary culture were submitted to acute (1 to 48 h) or chronic (3 days or more) stimulation with TSH (1 milliunits/ml) or EGF (25 ng/ml). Total RNA was extracted and subjected to Northern blot analysis. (—, no modulation; ↑, increased expression; ↓, decreased expression; ‡, increase followed by decrease).

| Clone | mRNA level | TSH | EGF |
|-------|------------|-----|-----|
| 5     | 3.7        | —   | ↑   |
| 3     | 4          | —   | ↑   |
| 2     | 1.5        | —   | ↑   |
| 4     | 1.4; 1.8   | NT  | ↑   |
| Hsp-90| 1.9; 2.7   | ↓   | ↑   |
| NAP-1 | <1         | ↓   | ↑   |
| 169   | 5          | —   | NT  |
| 45    | mRNA       | —   | NT  |
| EF-1α | 1.8        | —   | NT  |

* NT, not tested.
were modulated by the cAMP-dependent and -independent mitogenic pathways in dog thyrocytes in primary cultures. This could result from modified transcription, degradation, or both. Remarkably, clone 3 mRNA levels were increased in vivo but not in vitro by TSH. This discrepancy suggests that the in vivo situation does not necessarily match the in vitro stimulation. Whereas in vitro the cAMP-dependent pathway alone is directly activated by TSH or forskolin, the situation in vivo appears to be more complex. The presence of EGF and IGF-1 in the thyroid has indeed been described (14), and the TSH stimulation induced in vivo by the treatment with the antithyroid drugs could indirectly involve these growth factors or even other, undescribed factors. In other words, the effects observed in vivo could be the result of different interacting signaling components, which is not the case in vitro. To confirm clone 3 mRNA up-regulation by agents acting via tyrosine kinase receptors, we investigated the action of HGF, which is the most potent mitogen for our cells (31). HGF reproduced the effects of EGF, but the increase was less pronounced. As HGF has a weaker dedifferentiating action on thyrocytes than EGF, these results may suggest that clone 3 mRNA expression is inversely related to the differentiation state of the thyrocytes.

Among the 19 clones which were apparently differentially expressed in chronically stimulated thyroids, 7 (2, 3, 4, 5, 45, hsp90, and NAP-1) were regulated in vivo, as suggested by the screening, and in vitro by the various mitogenic cascades; three (101, 51, and IRK-2) were not detected by Northern blot analysis, four (16, 59, 137, and 166) led to uninterpretable results, and two (EF-1α, and 169) were not regulated.

In our thyrocytes, hsp90 mRNA expression was modulated by chronic treatments: positively by EGF, negatively by TSH and forskolin. As both these agents are mitogenic, it is difficult to relate this expression to proliferation as in some other systems (33–37). NAP-1 assembles the nucleosomes by mediating the formation of a histone octamer and transferring it to DNA (38, 39). In our cells, NAP-1 mRNA varied in a cyclical manner in response to TSH. This could correspond to the modulation of the histones, which are well known cell cycle-dependent genes. During the cell cycle of Balb/c 3T3 cells, the content of NAP-1 was found to change in parallel with histone synthesis (40). The modulation of its expression in our cells is compatible with this

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**FIG. 4.** Clone 3 mRNA levels in dog thyrocytes in primary culture. A, the cells were incubated with forskolin (Fo) (10⁻⁵ M) or EGF (25 ng/ml) for the indicated times. Total RNA was extracted and subjected to Northern blot analysis (cont, control). The corresponding acridine orange stainings are shown. B, the cells were incubated for 48 h with EGF (25 ng/ml) or HGF (50 ng/ml), alone or in combination with TSH (1 milliunit/ml). Total RNA was extracted and subjected to Northern blot analysis (cont, control). The lower part of the figure shows acridine orange staining of the gel.

**FIG. 5.** Clone 2 mRNA levels in dog thyrocytes in primary culture. The cells were incubated with EGF (25 ng/ml) + FCS (10%) for 3 days, or with TSH (1 milliunit/ml) for the indicated times. Total RNA was extracted and subjected to Northern blot analysis (cont, control). The lower part of the figure shows acridine orange staining of the gel.

**FIG. 6.** Hsp90 and NAP-1 mRNA regulation in dog thyrocytes in primary culture. The cells were incubated with forskolin (Fo) (10⁻⁵ M) for the indicated times. Total RNA was extracted and subjected to Northern blot analysis. The control lanes (CONT) refer to cells incubated further in control medium for 1 or 2 days. The lower part of the figure shows acridine orange staining of the gel.

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The entire protein sequences are shown schematically.

b Structure of clone 3 gene product

| ANK-like repeat |
|-----------------|
| NH | 1 2 3 4 5 |
| 31-amino acid repeats |

| consensus | G-TPLH-AA-G-CC-V-L-| GA-X-T | A | D |

Fig. 7. Sequence homologies of clones 5 and 3 gene products. The entire proteins are shown schematically. a, clone 5. Homologous regions with subdomains VIII and IX of different protein kinases are shown (accession numbers: 1, pir | A44499; 2, pir | JU9270; 3, sp | P22987; 4, sp | Q05512; 5, sp | P18653). The consensus line is given according to the following code: uppercase letters, invariant residues; lowercase letters, nearly invariant residues; o, positions conserving nonpolar residues; s, positions conserving small residues with near-neutral polarity (from 22); b, clone 3. The position and the alignment of the six 31-amino acid, ankyrin-like repeats are indicated. The consensus sequence is shown below.

The expression of a human NAP-related gene also increases rapidly in T cells after induction of cell proliferation, and mitogenesis was inhibited in cells treated with the corresponding antisense oligonucleotide (41).

On the basis of the in vitro mRNA regulation results, we selected first clones 5 and 3 for further characterization. Both were completely sequenced. Clone 5 protein sequence comparison with the data bases revealed ~30% identity with the protein kinase family. This large superfamily of homologous proteins contains a 250–300-amino acid kinase domain, composed of 12 conserved subdomains that fold into a common catalytic core structure (32). However, our clone lacks most of these consensus motifs: indeed, only subdomains VIII and IX are present (with an identity of ~80%). Subdomain VIII appears to play a major role in the recognition of peptide substrates, and contains several autophosphorylation sites, required for maximal kinase activity in many protein kinases. No clear ATP-binding site is identified as in the Bcr protein serine/threonine kinase (42) and in the human A6 tyrosine kinase (43). Clone 5 could represent a new example of these atypical kinases, and work is currently in progress to test this hypothesis.

Clone 3 protein sequence comparison with the data bases showed that this protein contains 6 ankyrin-like repeats. The ankyrin-like repeat is a 31–33-amino acid motif which consists of a TPLHLA core sequence and 8–10 other well conserved residues. These motifs are found in ankyrin and several other proteins, very heterogeneous with respect to their origin, function, intracellular localization, and number of repeats. These proteins include cell cycle control proteins (e.g. cdk10 gene product of Schizosaccharomyces pombe), putative integral membrane proteins (e.g. Notch gene product of Drosophila melanogaster, involved in cellular differentiation), transcription factors (e.g. NF-kB p105 precursor), and others (e.g. Ig-like molecules, virally-encoded proteins) (44). More recently, ankyrin repeats have been discovered in INK4 proteins, a family of cell cycle regulators characterized by their selective inhibition of cdk4 and cdk6 (45–47). Ankyrin-like repeats are involved both in interactions between distinct proteins and within a single molecule, and this can serve a variety of functions. The presence of 6 such motifs in clone 3 protein suggests that it might interact with other proteins, and these putative partners will be sought. We are now studying the possible role of clone 3 and clone 5 proteins in the proliferation and differentiation of the thyroid cell.

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