DARPP-32 (Dopamine and 3',5'-Cyclic Adenosine Monophosphate-Regulated Neuronal Phosphoprotein) Is Essential for the Maintenance of Thyroid Differentiation

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Coordination of events leading to differentiation is mediated by the concerted action of multiple signal transduction pathways. In general, the uncoupling of mechanisms linking differentiation to cell cycle exit is a hallmark of cancer, yet the identity and regulation of molecules integrating signal transduction pathways remains largely unknown. One notable exception is DARPP-32 (dopamine and cAMP-regulated neuronal phosphoprotein, molecular mass, 32 kDa), a third messenger that integrates multiple signaling pathways in the brain. Thyroid cells represent an excellent model for understanding the coupling of signal transduction pathways leading to both proliferation and differentiation. The cooperative action of IGF-I and TSH together, but not alone, enable thyroid cells to proliferate while maintaining their differentiated state. How signaling downstream from these molecules is integrated is not known. Here we show that DARPP-32 expression is targeted by TSH and IGF-I in thyrocytes. Significantly, dedifferentiated, tumoral, or Ras-transformed thyrocytes fail to express DARPP-32 whereas short interfering RNA-mediated silencing of DARPP-32 expression in normally differentiated thyroid cells results in loss of differentiation markers such as thyroid transcription factor 1, Pax8, thyroglobulin, and the Na/I symporter. Consistently, DARPP-32 reexpression in ras-transformed cells results in reactivation of the otherwise silent thyroglobulin and thyroperoxidase promoter. Thus, DARPP-32 is critical for the maintenance of thyroid differentiation by TSH and IGF-I, and loss of DARPP-32 expression may be a characteristic of thyroid cancer. Our results also raise the possibility that DARPP-32 may play a similar role in the maintenance of differentiation of a range of other cell types. (Molecular Endocrinology 19: 3060–3072, 2005)

CELL DIFFERENTIATION OCCURS as a result of the concerted activation of multiple signaling pathways in response to environmental stimuli. In general, differentiation occurs at the expense of proliferation and, as such, signaling pathways regulating proliferation must be coordinated with those promoting differentiation. Thyroid cells represent an excellent system because the main stimuli governing their function cooperatively promote both growth and differentiation. Differentiated thyroid cells express a number of final differentiation markers such as thyroglobulin (Tg), thyroperoxidase (TPO) and the Na/I symporter (NIS) the expression of which is governed by combinations of ubiquitous and thyroid-specific transcription factors such as thyroid-specific transcription factor (TTF)1, TTF2, and Pax8 (1). Differentiated thyroid cells in culture are dependent on the presence of TSH and insulin/IGF-I, which cooperate to promote both growth and differentiation (2, 3). Undifferentiated thyrocytes can also be maintained in culture in a medium depleted of serum, TSH, and IGF-I. Although cooperation of both TSH and IGF-I is required for correct thyrocyte function, the molecular mechanisms leading from the TSH and IGF-I membrane receptors to thyroid-specific transcription factors and consequent expression of final differentiation markers are not well understood. TSH binds its G protein-coupled receptor producing an increase in intracellular cAMP, which activates protein kinase A (PKA), and other pathways such as phosphatidylinositol 3-kinase (PI3K). IGF-I, through its tyrosine kinase receptor, activates both the PI3K and protein kinase C pathways. Additionally, there are indications suggesting that a convergence from both the TSH- and IGF-I signaling pathways might exist in the thyrocyte (4).

Given the importance of IGF-I and TSH signaling for thyroid cell function, a major question concerns
the identity of the molecules that play a key role in facilitating their cooperative action. Such mediators are important as messengers of hormone action, as signal integrators, and as therapeutic targets. An example of such “messengers or integrators” is provided by DARPP-32 (dopamine and cAMP-regulated neuronal phosphoprotein; molecular mass, 32 kDa) a molecule the expression of which was once thought to be restricted to dopaminergic neurons in specific brain areas where it acts as a molecular switch integrating signals from different membrane receptors. At least 10 signaling pathways are known to alter DARPP-32 function in the medium spiny neurons of the striatum (5) where its function and regulation have been well studied. Furthermore, diverse electrophysiological and biochemical alterations, as well as interesting behavioral peculiarities, which characterize DARPP-32 knockout mice, reflecting the importance of the protein (6). Thus, DARPP-32 is the only known molecule capable of alternatively inhibiting either protein phosphatase 1 (PP1) or PKA, both of which play crucial roles in most cell types.

Here we show that DARPP-32 is expressed in thyrocytes, its levels correlate with the degree of differentiation, and its expression is abolished with transformation. Importantly, silencing of DARPP-32 using short interfering RNA (siRNA) results in loss of the thyroid-specific differentiation markers and conversely DARPP-32 reexpression in Ras-transformed cells results in reactivation of the otherwise silent Tg and TPO promoters. Our results identify DARPP-32 as a critical factor for thyroid differentiation promoted by TSH and IGF-I.

RESULTS

DARPP-32 Is Expressed in Thyroid Cells

In the course of examining mRNAs that were differentially expressed between differentiated and undifferentiated thyroid cells, we isolated by RT-PCR a cDNA corresponding to DARPP-32, a protein that alternatively inhibits PP1 or PKA in dopaminergic neurons of specific brain regions. To confirm the unexpected observation that this gene is also expressed in thyroid, we performed Northern blots using total RNA extracted from the thyroid cell line FRTL-5, unrelated Rat1 fibroblasts, and fetal lung tissue as indicated using a DARPP-32 cDNA probe. The results (Fig. 1A) confirmed that DARPP-32 was expressed in the thyroid cell line FRTL-5, but not in unrelated Rat1 fibroblasts or fetal lung tissue. Similar results were observed using the PC Cl3 thyroid cell line (data not shown). To confirm that the gene was expressed in primary thyroid tissue, we made Western blots using a specific anti-DARPP-32 antibody and protein extracts from brain and thyroid tissue. The results confirmed that DARPP-32 is expressed in both thyroid and brain as well as the PC Cl3 thyroid cell line (Fig. 1B). Actin was used as a loading control. The high DARPP-32 expression levels found in cultured cells, as compared with the primary tissue, could be attributed to many reasons. For example, expression in the tissue could be restricted to some cells in certain follicles as reported for other thyroid proteins such as human apical iodide transporter (7) or could be due to natural negative regulators present in the whole animal and absent in the cultured cells.

Although in neurons DARPP-32 is located in the soluble fraction of the cytoplasm, in the parathyroid DARPP-32 was found in the membrane fraction where its target, PP1, is devoted to specific substrates (8). To understand whether in thyroid cells DARPP-32 is restricted to the membrane or soluble fractions, we fractionated cell extracts from the PC Cl3 thyroid cell line. Western blotting (Fig. 1C) re-
revealed that most of the protein was located in the soluble cytoplasmic fraction as in neurons and was absent from the membrane fraction in contrast to parathyroid cells. The small amount observed in the nuclear fraction is due to small amount of cytoplasm associated with the nuclei (see also some actin) when fractionated by the Dignam method. Furthermore, microscopic observation of the cells stained with anti-DARPP-32 antibodies reveals that most of the protein is cytoplasmic (see Fig. 4B) with the background nuclear staining being also found in cells where DARPP-32 is undetectable by Western.

**DARPP-32 Expression Correlates with Thyroid Differentiation**

In our initial RT-PCR experiments we found DARPP-32 highly expressed in normal, differentiated thyroid cell lines whereas it was absent in the nondifferentiated Ha-Ras and K-Ras-transformed FRTL-5 thyroid cell lines (9), as well as in the undifferentiated human thyroid tumoral cell line (FRO) (10). It was also absent from the undifferentiated thyroid-derived cell line FRT, which manifests the polarized epithelial phenotype but does not express any of the thyroid functional properties (11). Northern blotting using a DARPP-32 probe on total RNA derived from these cell lines confirmed the absence of DARPP-32 mRNA in the nondifferentiated thyroid cell lines (Fig. 2A). The results revealed that DARPP-32 mRNA levels were decreased in parallel with the degree of undifferentiation. Compared with the high levels of DARPP-32 mRNA found in normally growing differentiated FRTL-5 cells, the Ha-ras transformed FRTL-5 cells had barely detectable levels, and DARPP-32 mRNA was undetectable in both the K-ras-transformed FRTL-5 cells (see below) and in the FRT cell line. These results suggested that when differentiation is compromised by transformation, DARPP-32 mRNA expression is silenced. Remarkably, DARPP-32 mRNA is also absent in the nontransformed, but undifferentiated, FRT thyroid cell line, reinforcing the correlation between lack of DARPP-32 expression and lack of differentiation. In agreement with this, Fig. 2B shows that DARPP-32 protein levels were also undetectable in the FRT, the K-ras/FRTL-5, and the FRO thyroid cell lines and were severely reduced in the Ha-ras-transformed FRTL-5 cells compared with the normal FRTL-5 or PC Cl3 thyroid cells. Recombinant DARPP-32 expressed in bacteria was used as a positive control.

Although lack of differentiation in the tumor-derived FRO and Ras-transformed thyroid cell lines correlates with suppression of DARPP-32 expression, loss of DARPP-32 expression could be a secondary event associated with transformation. Nevertheless, the correlation between DARPP-32 silencing and transformation, together with the fact that the nontransformed but undifferentiated thyroid cell line FRT is devoid of DARPP-32 expression, raised the possibility that DARPP-32 might play a role in thyroid differentiation. To further confirm that DARPP-32 expression correlates with differentiation in nontransformed thyroid cells, DARPP-32 expression was compared between quiescent, undifferentiated PC Cl3 cells cultivated for 8 d in 4H medium and differentiated PC Cl3 cells grown in the presence of TSH and IGF-I in 6H medium. In 4H medium cells are quiescent, proliferation is minimal, and final differentiation markers (such as Tg and NIS) are not expressed, whereas cells proliferating in 6H medium do express final differentiation markers. Total protein extracts from cells cultivated in 4H or 6H medium were subjected to Western blotting and the results are shown in Fig. 2C. Untransformed, quiescent thyroid cells cultivated in 4H medium and not expressing differentiation markers show greatly reduced DARPP-32 protein levels compared with differentiated thyroid cells growing in 6H medium (5- to 8-fold). Thus again, DARPP-32 expression levels correlate with differentiation, suggesting that a pre-
cise control of its activity may be required for proper function and differentiation of thyroid cells. The extent of induction of DARPP-32 expression in 6H medium varied between different experiments and reflected differences in the degree of confluence at the onset of starvation in 4H medium (data not shown): slightly higher confluence at the onset of starvation results in leaky expression of differentiation markers, such as NIS, Tg, and DARPP-32, that partially masks the induction by 6H medium. This observation again emphasizes the parallel previously established between DARPP-32 levels and expression of differentiation markers. Furthermore, DARPP-32 mRNA or protein levels in cells cultivated in 4H medium are low but detectable and at least 20 times higher than in Ha-Ras-transformed cells where DARPP-32 is hardly detectable (data not shown).

**DARPP-32 Is Required to Maintain Thyroid Cell Differentiation**

Our results show that TSH and IGF-I stimulate DARPP-32 expression in thyroid and that DARPP-32 expression strongly correlates with differentiation, raising the possibility that DARPP-32 plays a role in TSH and IGF-I-mediated thyrocyte differentiation. We used siRNA to deplete differentiated thyrocytes of DARPP-32 and asked whether the levels of the differentiation markers were affected. A modified pSuper vector expressing green fluorescent protein (GFP) and either a DARPP-32-specific siRNA (pSGD), or a control luciferase-specific siRNA (pSGL), were transfected into PC Cl3 thyroid cells. Expression of DARPP-32, Tg, and NIS was examined by immunofluorescence (data not shown). The results indicated that DARPP-32 and Tg expression were significantly reduced in the GFP-positive cells transfected with the pSGD vector and unaffected in control cells transfected with the vector pSGL (see confocal photographs in Fig. 4). This was confirmed by Western blotting of total protein extracts of cells transfected with either interference vector as described above using anti-NIS antibodies to monitor differentiation. The results are presented in Fig. 3A. Actin was used as a loading control whereas the expression of GFP reflects the efficacy of transfection. Compared with the levels in the pSGL control-transfected cells, DARPP-32 expression was reduced in pSGD-transfected cells (DARPP-32 silenced) to approximately 30% of the control levels. Importantly, NIS levels in the pSGD (DARPP-32 siRNA)-transfected cells were 20% compared with the control pSGL-transfected cells. Note that any reduction in DARPP-32 expression will only occur in those cells that are transfected and GFP positive. The efficiency of transfection was similar in the DARPP-32 or Luc-control siRNA-transfected cells as shown by the expression levels of the GFP protein as well as by microscopy (data not shown). Note also that the slight increase in the GFP signal observed in the pSGD-transfected cells arises in part due to a marginal increased loading of this track as evidenced by a slightly increased actin signal.

Expression of thyroid final differentiation markers such as Tg or NIS is dependent on the transcription factor Pax8. We examined whether Pax8 levels were affected by the interference with DARPP-32 expression. Significantly, Western blotting using anti-Pax8 antibodies (Fig. 3B) indicated that on depletion of DARPP-32, Pax8 protein levels were also reduced to 20–30% of the control levels. The results are consistent with a model in which DARPP-32 depletion results in Pax8 depletion and consequent loss of the final differentiation markers Tg and NIS.

The results obtained using the pSuper-derived vectors were confirmed using DARPP-32-specific siRNA. Importantly, the efficacy of transfection using siRNA was much higher than with the pSuper-derived vectors and, compared with cells transfected with a non-silencing siRNA control, by 24 h post transfection virtually all DARPP-32 was eliminated from the cells as determined by Western blotting (Fig. 4A). DARPP-32 levels remained very low 3 d after transfection but by d 7 normal levels were recovered. We also monitored several differentiation markers by Western. Strikingly, depletion of DARPP-32 led to decreased levels of TTF1, as well as Tg and NIS, and the levels of these factors also recovered over time in parallel with DARPP-32. Actin was used as a loading control. Immunofluorescence assays using anti-DARPP-32 and anti-Tg together with confocal imaging confirmed the results of the Western analysis. Whereas both DARPP-32 and Tg were expressed robustly in control cells, siRNA-mediated depletion of DARPP-32 led to a
A dramatic reduction in both DARPP-32 and Tg expression within 1 d, the levels of both proteins recovering over time. Note that by d 2 some cells expressing DARPP-32 were already visible and these cells also expressed Tg with the proportion of cells expressing both markers increased with time. Thus, the results

**Fig. 4.** DARPP-32 Depletion by siRNA Interference Causes Lack of Differentiation in Thyroid Cells

A, TTF1, Tg, and NIS are specifically depleted by the DARPP-32 siRNA. Western blot of total protein extracts from cells transfected with the Control siRNA (C) or the anti-DARPP-32-specific siRNA and collected on d 1–7. Control extracts correspond to d 1. B, Confocal images of cells transfected with the siRNAs Control or DARPP-32 specific. In each picture the DAPI staining shows total number of cells in the field; green is anti-DARPP-32 staining, and red is anti-Tg staining. Cells transfected with the control siRNA show normal levels of both DARPP-32 and Tg. DARPP-32 depleted cells collected from d 1–5 show the lack of both DARPP-32 and Tg and progressive recovery of both by d 5. The arrows on d 2 mark cells with a parallel recovery of both DARPP-32 and Tg expression. DAPI, 4',6-Diamidino-2-phenyindole.
obtained using both the pSuper and siRNA are consistent with DARPP-32 playing a key role in the maintenance of differentiation.

Reintroduction of DARPP-32 into Ras-Transformed Cells Overcomes Their Inability to Activate the Tg and TPO Promoters

Upon DARPP-32 depletion, thyroid cells lose expression of differentiation markers such as Tg, suggesting that DARPP-32 is required for full thyroid cell differentiation. If DARPP-32 is required for Tg expression, then reintroduction of DARPP-32 in Ras-transformed thyroid cells that are devoid of DARPP-32 and Tg expression should enable them to reactivate the Tg promoter. Ha-ras-transformed cells were transiently transfected with the expression vector pEGFP-DARPP-32 and the fusion Tg promoter-luciferase reporter to quantify promoter activity (Fig. 5A). Consistent with previous reports (9) ectopic expression of TTF1 in these cells was not sufficient to activate the Tg promoter. However, ectopic expression of DARPP-32 resulted in activation of the Tg promoter (see a 2.5-fold increase in luciferase activity) consistent with DARPP-32 playing a role in differentiation, and TTF1 coexpression resulted in a marginal increase over the induction obtained by expression of DARPP-32. Previous studies (12) suggested that the equilibrium between kinases and phosphatases necessary to maintain TTF1 active is broken in Ha-ras-transformed thyroid cells as revealed by an altered phosphorylation pattern of TTF1. Therefore, exogenous DARPP-32 expression may increase the TTF1 levels and rescue the kinases/phosphatases equilibrium in Ha-ras-transformed cells rendering enough active TTF1 to promote Tg promoter activity. Figure 5B shows similar results obtained with the TPO promoter, another classical differentiation marker. TPO promoter is silent in Ha-ras-transformed thyrocytes, and we show that after reexpressing DARPP-32 its activity increases 2-fold similarly to the Tg promoter. Coexpression of TTF1 and DARPP-32 only results in a marginal increase over that produced by DARPP-32 alone. These results further confirm that DARPP-32 plays a crucial role in signal transduction pathways leading to thyroid differentiation.

TSH and IGF-I Independently Up-Regulate DARPP-32 Levels

Because TSH and IGF-I are the most physiologically relevant stimuli for thyroid function and we have shown that DARPP-32 is required for thyroid differentiation, we wished to study whether these stimuli targeted DARPP-32 expression. Undifferentiated PC Cl3 cells cultivated in 4H medium for 8 d were treated with TSH and/or IGF-I for 24 h. Western blotting of total protein extracts (Fig. 6) revealed that TSH induced DARPP-32 expression 5- to 8-fold above basal levels after 24 h treatment. Similar results were obtained with IGF-I. Simultaneous addition of TSH and IGF-I resulted in a similar induction to that observed using either TSH or IGF-I. Actin was used as a loading control. Quantification of the results obtained in three independent experiments as is shown at the bottom graphics demonstrates that the extent of induction is similar with either or both stimuli.

Thus both TSH or IGF-I independently exert maximal DARPP-32 induction, and DARPP-32 is a common target for multiple signaling pathways involved in thyroid function or differentiation.

Fig. 5. DARPP-32 Reintroduction in H-ras-Transformed Cells Overcomes Their Inability to Activate the Tg and TPO Promoters

Ha-ras/FRTL-5 cells were transfected with the Tg (A) or TPO (B) promoter fused to the luciferase reporter and/or the TTF1 expression vector pTTF1 and/or pEGFP-N1 or the DARPP-32 expression construct pEGFP-DARPP-32. Relative luciferase activity normalized with Renilla was measured 72 h after transfection. Promoter activity is expressed as fold induction over basal level (± 1) of the cells transfected with either the pTg (A) or pTPO (B) construct alone. The results represent the mean ± so of three independent experiments. The bottom panels show the expression levels of ectopically expressed DARPP-32 in one representative experiment. EGFP, Enhanced GFP.
The PKA and PI3K Pathways Are Required for DARPP-32 Induction by TSH and IGF-I, Respectively

To unravel critical components of the signaling pathways from TSH or IGF-I to DARPP-32, we asked whether specific protein kinase inhibitors could affect the TSH- or IGF-I-mediated induction of DARPP-32 expression. We first used H89, an inhibitor of PKA. PC Cl3 cells cultivated in 4H medium for 8 d were pre-treated with 10 μM H89 for a minimum of 30 min before addition of thyrotropin or IGF-I for 24 h. TSH and IGF-I in the absence of the inhibitor exerted an induction similar to that shown in the previous figures (data not shown). In contrast, H89 treatment abolished both TSH and IGF-I-mediated induction of DARPP-32 (Fig. 7A). Treatment with H89 alone did not modify basal levels of DARPP-32 after 30 min treatment (data not shown) or after 24 h (compare first and last lanes).

The PI3K pathway plays an important role in thyroid cell signal transduction downstream of IGF-I (13), and it has been reported to cross talk with TSH signaling (14) (15). To determine whether PI3K was important for DARPP-32 regulation, PC Cl3 cells cultivated in 4H medium for 8 d were pre-treated with 10 μM H89 for a minimum of 30 min before addition of thyrotropin or IGF-I for 24 h. TSH and IGF-I in the absence of the inhibitor exerted an induction similar to that shown in the previous figures (data not shown). In contrast, H89 treatment abolished both TSH and IGF-I-mediated induction of DARPP-32 (Fig. 7A). Treatment with H89 alone did not modify basal levels of DARPP-32 after 30 min treatment (data not shown) or after 24 h (compare first and last lanes).

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TSH and IGF-I Promote DARPP-32 Phosphorylation at Thr34

So far we could block differentiation by depleting DARPP-32 in thyroid cells and conversely promote differentiation by reintroduction of DARPP-32 in cells poorly expressing the protein. Moreover, TSH and IGF-I, the most physiologically relevant stimuli for thyroid function, induced DARPP-32 accumulation. DARPP-32 function depends on its phosphorylation status: when phosphorylated on Thr75 DARPP-32 inhibits PKA while phosphorylated on Thr34 inhibits PP1, these phosphorylation events being mutually exclusive (i.e. phospho Thr34 DARPP-32 cannot be
phosphorylated at Thr75 and vice versa). We were unable to detect P-Thr75 DARPP-32 in our cells but could easily detect the P-Thr34 form that inhibits PP1 (Fig. 8). Furthermore, we observed that both TSH and IGF-I independently induced phosphorylation of DARPP-32 at Thr34 (Fig. 8A). P-Thr34-DARPP-32 induction by TSH peaks at 12 h of treatment (5- to 6-fold) and by 24 h is approximately 3-fold relative to actin levels. IGF-I induction of P-Thr34-DARPP-32 was also 3- to 4-fold at 12 h and still increased up to 6-fold at 24 h (Fig. 8B). TSH induction of P-Thr34 was mediated via PKA because it was prevented by its inhibitor H89 (Fig. 9A). Induction of P-Thr34 at 12 h by IGF-I was unaffected by H89, but the phosphorylation of DARPP-32 was transient with no signal being apparent by 24 h, in contrast to cells not treated with the inhibitor (see Fig. 8B). The PI3K inhibitor LY294002 did not affect TSH-induced phosphorylation of DARPP-32 (Fig. 9B). Any effect of LY294002 on IGF-I-induced phosphorylation of DARPP-32 was difficult to assess given our observation that this inhibitor abolishes basal and IGF-I induced DARPP-32 expression (see Fig. 7B). We were consequently unable to detect P-Thr34 DARPP-32 in the presence of LY294002 alone or with IGF-I. In the absence of any inhibitor, IGF-I and TSH induced DARPP-32 phosphorylation as expected (data not shown). These data suggest that both TSH and IGF-I induce DARPP-32 expression and, through phosphorylation of DARPP-32 at Thr34, convert it into a potent inhibitor of PP1.

DISCUSSION

DARPP-32 is a bifunctional molecule the expression of which was once believed to be restricted to dopaminergic neurons in discrete regions of the brain where its function has been extensively studied. Its importance in neurons is highlighted by the fact that more than 10 different signaling pathways alter this molecule, and its function has been implicated in a number of diseases including Parkinson, Huntington, schizophrenia, hyperactivity and attention deficit syndrome, and long-term depression (18). Neuronal DARPP-32 has been shown to mediate the effects of dopamine, cocaine, serotonin, fluoxetine (Prozac), and caffeine and a range of other molecules. Here we report the finding that DARPP-32 is expressed in normal thyroid gland and thyroid cell lines. More importantly, we provide four lines of evidence that DARPP-32 is essential for the maintenance of thyroid cell differentiation. First, thyroid cells kept undifferentiated in 4H medium possess low basal DARPP-32 levels, which are inducible by TSH or IGF-I as are other differentiation markers. TSH and IGF-I induction of thyroid differentiation has been widely documented before (19, 20, 21, 22) although this is the first report of DARPP-32 expression in these cells. Second, there is a good correlation between DARPP-32 levels and the degree of differentiation. Thus, DARPP-32 is absent in...
tumoral (FRO) or Ras-transformed thyroid cell lines and in the undifferentiated but nontransformed thyroid cell line FRT. Third, siRNA-mediated silencing of DARPP-32 leads to a dramatic inhibition of expression of the key thyroid transcription factors, Pax8 and TTF1, and probably as a consequence of thyroid-specific differentiation markers such as Tg and NIS. Fourth, reintroduction of DARPP-32 in Ha-ras-transformed thyroid cells enables them to activate the otherwise silent Tg promoter.

TSH and IGF-I cooperate to promote thyrocyte proliferation and function (3, 23). We demonstrate that both TSH and IGF-I induce total DARPP-32 accumulation and also its phosphorylation at Thr34, thereby converting it into a potent inhibitor of PP1. Phosphorylation of DARPP-32 on Thr34 prevents its phosphorylation at Thr75, a form of the protein that inhibits PKA. The lack of P-Thr75 DARPP-32 in thyroid cells possibly reflects the importance of an active PKA pathway for these cells to survive or differentiate. Moreover, the mechanisms by which TSH and IGF-I induce accumulation of total DARPP-32 are different: TSH induction occurs through mRNA accumulation, whereas IGF-I induction appears to be posttranscriptional (our manuscript in preparation).

Conversely, we have also tested the hypothesis that DARPP-32 may increase TSH and IGF-I receptor levels giving rise to a positive feedback loop involving TSG/IGF-I, their receptors, and DARPP-32. We detected a significant decrease in IGF-I receptor levels when silencing DARPP-32 (our unpublished observations). However, under our conditions the quality of the TSH-R antibody does not allow us to discard a hypothetical decrease of the TSH-R in cells transfected with DARPP-32-specific siRNA. Nevertheless it is generally accepted that failures in signaling intermediates from the TSH-R are responsible for the lack of differentiation. Accordingly, in a recent study by Di Lauro and associates (24), Ha-ras-transformed thyrocytes are shown to differentiate in response to TSH only when Ras is expressed at low dose but not at high dose. It would therefore be particularly interesting to determine whether DARPP-32 expression levels parallels Ras dose in their system.

Proliferation of thyroid cells, on the other hand, does not seem to be controlled by DARPP-32 levels. We did not observe an effect of DARPP-32 on thyroid cell proliferation neither when we depleted it from normally growing thyroid cells nor on its reintroduction into Ha-ras-transformed cells. Moreover, thyroid cells with proliferation rates varying from the very high (Ras-transformed and tumoral) to the nonproliferative thyroid cells cultivated in 4H medium have undetectable or very low DARPP-32 levels.

Malignant transformation is often characterized by dedifferentiation. Consistent with this, Ras-mediated transformation of thyroid cells or naturally occurring thyroid tumor cell lines exhibit a loss of differentiation markers such as Tg and NIS. Our results presented here indicate that DARPP-32 expression is also lost in transformed and tumoral thyroid cells and may represent a useful marker for the progression of thyroid carcinoma. In contrast, in gastric cancers (25) and some common adenocarcinomas DARPP-32 is over-expressed (26) compared with their normal untransformed parental cell types. Future research will uncover any potential role of DARPP-32 in cancer progression. Nevertheless, the evidence suggests that DARPP-32 may lie at the nexus of multiple signaling pathways that modulate or maintain the differentiation state of a given cell type.

IGF-I-dependent induction of DARPP-32 in thyroid cells, mediated by activation of PI3K, is reminiscent of the PI3K-dependent induction of DARPP-32 in neurons by brain-derived neurotrophic factor acting through its tyrosine kinase receptor (27), and it may be a general theme that receptor tyrosine kinases in other cell types may use this signaling pathway to regulate DARPP-32 expression. Indeed, although we have focused here on the effects of TSH and IGF-I on thyroid, TSH and IGF-I receptors are also expressed in neurons (28, 29), and, potentially, neuronal DARPP-32 levels could be subjected to similar regulation. This may be particularly important for understanding the link between metabolic and psychological effects of drugs targeting neuronal function. Thus, DARPP-32 may be viewed as a third messenger of hormones, growth factors and neurotransmitter action. In thyroid, the role of DARPP-32 appears to be in mediating the effects of TSH and IGF-I in differentiation. Thus, in the absence of TSH or IGF-I, or on depletion of DARPP-32, we observe a loss of expression of the thyroid differentiation markers Tg and NIS. Iodination of Tg residues is essential for the differentiated function of the thyrocyte and depends on functional NIS at the membrane. The expression levels of NIS at the membrane directly correlate with iodide uptake both in thyroid and nonthyroid cells (30, 31). We have examined NIS expression at the membrane after DARPP-32 silencing in thyrocytes, and we observe a direct correlation between the levels of DARPP-32 and the membrane population of NIS (data not shown). The expression of these final differentiation markers depends on the activity of “teams” of cell-specific and ubiquitous transcription factors (1), the most important among them being TTF1, TTF2, and Pax8. Pax8 has been previously shown to be required for differentiation of competent endoderm into follicular thyroid cells producing thyroid hormones (32), and our results suggest that DARPP-32 is necessary to maintain the Pax8 expression/activity required for differentiation promoted by TSH and IGF-I. We also show that DARPP-32 expression is necessary to maintain adequate levels of active TTF1. Ha-ras-transformed thyroid cells contain low levels of inappropriately phosphorylated, and therefore inactive, TTF1 (12). Reintroduction of DARPP-32 capable of inhibiting PP1 might be therefore necessary for correct TTF1 and Pax8 phosphorylation and activity and
therefore reexpression of DARPP-32 in Ha-ras-transformed thyrocytes results in reactivation of the Tg and TPO promoter. As far as we are aware, this is the first demonstration that DARPP-32 as an integrator of signal transduction pathways plays a key role in differentiation. Our data could be particularly relevant in adipocytes where it has been reported that DARPP-32 levels dramatically increase upon 3T3-L1 differentiation toward adipocytes (33).

We suggest for thyroid cells the model depicted in Fig. 10. In this model, the principal role of DARPP-32 (Fig. 10B) is to inhibit the activity of PP1 and thereby stabilize the TSH and IGF-I-induced phosphorylation and activation of key transcription factors. Basal levels of DARPP-32 are required to amplify immediately the TSH and IGF-I effects. TSH and IGF-I stimulation (via PKA and PI3K, respectively) will induce phosphorylations affecting the levels and activity of transcription factors (Pax8, TTF1), which will turn on transcription of differentiation markers (Tg, NIS). TSH/IGF-I-induced accumulation and phosphorylation of DARPP-32 at Thr34 turns off the general phosphatase PP1 maintaining high levels of phosphorylated transcription factors, which prolongs the initial phosphorylation events allowing accumulation of differentiation markers. The effects of TSH and IGF-I would then be further amplified by their ability to induce DARPP-32 expression. By contrast, in cells lacking DARPP-32 expression (Fig. 10A), unrestricted PP1 activity will reverse any TSH- and IGF-I-induced phosphorylation of TTF1 and Pax8. Any effect of TSH and IGF-I signaling would therefore be transient and insufficient to support the accumulation of active Pax8 and TTF1, and full differentiation would not be possible.

**MATERIALS AND METHODS**

**Cell Culture**

Cell lines used in this work were: rat thyroid follicular FRTL-5 (34) (ATCC CRL 8305; American Type Culture Collection, Manassas, VA), Ha-ras- and K-ras-transformed FRTL-5 cells (35), rat PC Cl3 (36), rat FRT (11), and human FRO (10). The cells were cultured in Coon’s modified Ham’s F-12 medium supplemented with 5% donor calf serum, glutamine, antibiotics, and a six-hormone mixture (1 nM TSH, 10 μg/ml insulin, etc.).

**Fig. 10. A Model for the Role of DARPP-32 in Thyroid**

In thyroid cells, phosphorylation of transcription factors (P-TFs) such as Pax8 and TTF1 in response to TSH and IGF-I signaling is required for their activation and consequently for their ability to activate transcription (Tx) of differentiation specific genes. In tumoral (FRO), Ras-transformed, or undifferentiated thyroid cells (FRT) lacking DARPP-32 (panel A), the ability of TSH and IGF-I to promote phosphorylation of transcription factors is largely reversed by the action of PP1. Consequently, the cells are undifferentiated. By contrast, in differentiated thyroid cells expressing DARPP-32 (panel B), the presence of active DARPP-32 (phosphorylated at Thr34) will lead to inhibition of PP1 and consequently IGF-I and TSH signaling will lead to the stable phosphorylation and activation of transcription factors that promote the expression of differentiation genes (Tg, NIS, etc.). In quiescent thyroid cells the low level of DARPP-32 expression observed should allow IGF-I and TSH to initiate the differentiation program, whereas the ability of IGF-I and TSH to increase DARPP-32 expression and activity will then amplify the prodifferentiation signals.
10 ng/ml somatostatin 10 μg/ml transferrin, 10 μM hydrocortisone, and 10 ng/ml glycyrl-L-histidyl-L-lysine acetate). The effect of TSH and IGF-I was studied by starving nearly confluent cells of TSH, IGF-I, and serum or both from Calbiochem, La Jolla, CA (and maintained for 24 h). Appropriated controls were established (i.e., 4 μg/ml inhibitor for 30 min, 4 μg/ml for 24 h, 4 μg/ml TSH). Tissue culture media and bovine TSH were purchased from Sigma Chemical Co. (St. Louis, MO). IGF-I was obtained from Preprotech (Rocky Hill, NJ). Donor calf serum was purchased from Life Technologies, Inc. (Gaithersburg, MD). The medium was changed every 2 d.

RNA Extraction and Northern Blot Analysis

Total RNA was isolated by the guanidinium-thiocyanate-phenol method (37) from the indicated cell lines or tissues after the indicated treatments. Total RNA (20 μg) was separated in 1% agarose gels containing 2.2 μM formaldehyde. RNA was blotted onto Nytran filters (Schleicher & Schuell, Keene, NH) as suggested by the manufacturer. Methylene blue staining of the blots revealed the integrity of the RNA and the presence of equal amounts in each lane. Hybridization and washing were carried out with a rat cDNA DARPP-32-specific probe kindly donated by Dr. Greengard (Laboratory of Molecular and Cellular Neuroscience, Rockefeller University, New York, NY) and labeled by Random oligo priming.

Protein Extraction, Western Blot, and Immunodetection

Total protein extracts were obtained by scraping the cells in RIPA buffer. Fractionated (cytoplasmic, nuclear) protein extracts from the indicated cell lines were obtained as described in Ref. 38; membrane fractions were isolated as described in Ref. 39. Protein extracts (20 μg) were separated in 6–12% SDS-PAGE gels and immunodetected after Western blotting. Antitotal DARPP-32 and anti-P Threo 34-DARPP-32 in 6–12% SDS-PAGE gels and immunodetected after Western blotting. Antitotal DARPP-32 and anti-P Threo 34-DARPP-32 were incubated with the nonregulated proteins. Equal protein loading was first monitored by Bradford assays and after transfer by detection of the nonregulated proteins, cdk5 or β-actin.

Constructs

A cytomegalovirus-GFP-PolyA fragment digested with Asel/MluI out of the pEGFP-C3 vector (CLONTECH Laboratories, Inc., Palo Alto, CA) previously deleted of the multiple cloning site by BamHI/BglII digestion and religation was cloned into the Smal site of pSuper and pSuper-Neo plasmids (Oligogene) generating the intermediate plasmid pSuperGFP (pSG) and the final construct pSuperGFP-Luc (pSGL). The following oligonucleotide sequences were designed and purchased from QIAGEN (UK) to synthesize the specific DARPP-32 RNAi: Oligo1: 5′-GAT CCC CAA GTC GAGGAG ACC CAA CCT TCA AAG GAG GGT GGT GTC CTT CCA TTC TTT TTG GAA A-3′ and oligo 2: 5′-AGG TTT TCC AAA AAA AGT CGA AGA GCA CCA ACC TCT CTT GAA GGT GTC TCT TCT ACT TGG G-3′; the oligos were annealed and phosphorylated using polynucleotide kinase from Promega, and transfected into cells (plated at 104 cells per well the previous day) using Oligofectamine (Invitrogen, San Diego, CA) and followed by autoradiography. Equal protein loading was first monitored by Bradford assays and after transfer by detection of the nonregulated proteins, cdk5 or β-actin.

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Transfection

PC13 cells were transfected while plated using Fugene (Roche Molecular Biochemicals, Indianapolis, IN) and following manufacturer suggestions. For microscopy and Western analysis of the siRNA, cells were transfected on cover slips at 104 cells per well in 24-well dishes. In every experiment 12 wells were transfected with the control construct pSGL containing the sequence to synthesize the luciferase siRNA and 12 wells were transfected with the construct pSGL containing the sequence for DARPP-32 siRNA and above described. Maximum GFP expression was reached 5–6 d after transfection and monitored directly using the green filter under the fluorescence microscope. Alternatively, siRNA oligonucleotides, either control or DARPP-32 specific, were transfected into cells (plated at 104 cells per well the previous day) using Oligofectamine (Invitrogen, San Diego, CA) and following manufacturer’s instructions. The medium was changed 24 h later and every 2 d afterward. Cells transfected directly with the siRNAs were collected on d 1–7 after transfection to analyze the expression of DARPP-32, Tg, NIS, etc. by immunofluorescence or Western blotting.

Indirect Immunofluorescence

Cells in cover slips were washed three times and fixed in Methanol at −20 C for 10 min; washed again and blocked with PBS containing 5% donor calf serum and 0.05% Tween for 1 h at room temperature, incubated with anti-DARPP-32 or anti-Tg antibody for 20 min at R/T, washed in PBS-Tween three times for 5 min, incubated with the secondary antibody Alexa 488 or Texas Red conjugated, respectively, washed again three times with PBS-Tween and mounted on Vectashield containing 4’,6-diamidino-2-phenylindole (Vector Laboratories, Inc., Burlingame, CA). Cells were observed under epifluorescence (Axiohot; Carl Zeiss, Thornwood, NY) using ×63 magnification on oil immersion objective or confocal microscopy (Leica Corp., Deerfield, IL). Images were recorded in a Olympus DP70 camera (Olympus Corp., Lake Success, NY) using DP70-BSW software.
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