Loss of Proliferative Potential during Terminal Differentiation Coincides with the Decreased Abundance of a Subset of Heterogeneous Ribonuclear Proteins

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Abstract. The decrease in abundance of a subset of highly conserved basic nuclear proteins is established to correlate with the loss of proliferative potential in association with the process of terminal differentiation in murine mesenchymal stem cells and human keratinocytes. These proteins, designated P²Ps for proliferation potential proteins, have apparent molecular masses of 30–40 kD, are associated with the 30–40S substructures of nuclear hnRNP complexes, and are recognized by antibodies made against core proteins of hnRNP particles. They also share an epitope in common with heat shock protein-90 (hsp90) and are recognized by two mAbs against hsp90. Two-dimensional electrophoretic Western blots furthermore show that P²Ps make up a subset of hnRNP proteins. Cells that possess these proteins express the potential to proliferate whether or not they are traversing the cell cycle. These include rapidly growing cells, reversibly growth-arrested cells, and nonterminally differentiated cells. In contrast, cells that have irreversibly lost their proliferative potential, such as terminally differentiated cells, show a marked reduction in the abundance of P²Ps as determined by immunodetection on Western blots. A correlation, therefore, exists between the presence of this subset of nuclear proteins and the proliferative potential in two cell types. These results raise the possibility that as a subset of hnRNP proteins, P²Ps may mediate posttranscriptional control of the processing of specific RNAs required for cell proliferation.

MAMMALIAN cell differentiation is associated with complex molecular changes involving both the activation and repression of multiple genes (25, 26). One of the important biological processes associated with differentiation is the irreversible loss of proliferative potential that occurs during a specific terminal event in differentiation (23, 35). Many cell types including hematopoietic, muscle, and neural cells undergo terminal differentiation as do adipocytes and epithelial cells (8, 35, 41). To better understand the phenomenon of differentiation-associated loss of proliferative potential, this process has been studied in murine 3T3 T mesenchymal stem cells (23, 35, 36) and normal human keratinocytes (24, 41). In 3T3 T cells, both nonterminal and terminal states of differentiation have been defined and the transition from the nonterminal to terminal state has been experimentally characterized (3, 9, 23, 34). Additionally, the biochemical changes specifically associated with the loss of proliferative potential during terminal differentiation have been clearly distinguished from those resulting from nonterminal differentiation per se (36).

The current research was initiated to study changes in the expression of a variety of antigens during the process of nonterminal and terminal differentiation in murine 3T3 T cells and normal human keratinocytes by Western blotting procedures. These assays demonstrated that the abundance of a group of 30–40-kD proteins detected by monoclonal antibodies against both heterogeneous ribonuclear (hnRNP)[1] core proteins and heat shock protein-90 were selectively reduced when cells lost their proliferative potential in association with terminal differentiation. In contrast, these proteins, designated P²Ps for proliferation potential proteins, were present in relative abundance in cells that retained their ability to grow. Additional biochemical studies support the conclusion that P²Ps represent a subset of hnRNP proteins that could serve a regulatory role in the processing of RNAs that are important in determining a cell's proliferative potential.

Materials and Methods

Cell Culture: Murine Mesenchymal Stem Cells

The BALB/c 3T3 T mesenchymal stem cell line and its derivation have pre-

1. *Abbreviations used in this paper*: CEPH, a human plasma fraction; hnRNP, heterogeneous ribonuclear proteins; HP, human plasma; hsp90, heat shock protein, 90 kD molecular mass; P²P, proliferation potential proteins.

viously been described (4). Rapidly growing stock cultures of these cells were maintained in DME containing 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a 5% CO_2 atmosphere.

To reversibly arrest the growth of 3T3 T mesenchymal stem cells due to serum deprivation, they were plated at a density of 4×10^3 cells/cm^2 in DME containing 10% FBS and after 12–24 h the cells were washed with DME and refed DME containing 0.5% FBS and maintained therein for 3–5 d.

3T3 T mesenchymal stem cells can also be induced to undergo differentiation into adipocytes via a process that involves three steps: (*a*) predifferentiation growth arrest; (*b*) nonterminal differentiation; and (*c*) terminal differentiation. This process occurs in a progressive manner during 6–10-d interval after low density, rapidly growing cells are cultured in heparinized DME containing 25% human plasma (DME/HP) (35). Predifferentiation growth arrest and nonterminal differentiation are also induced when cells are cultured in heparinized DME containing a human plasma fraction designated CEPH (DME/CEPH) as described previously (35). That is, in this medium the terminal event in the differentiation process is repressed (34).

The kinetics of this process are dependent on the substrate used to culture the cells (35), but in general, within 3 d after addition of DME/HP or DME/CEPH, most cells undergo growth arrest at the predifferentiation state. Soon thereafter, most of such cells become nonterminally differentiated and thereafter when cultured in DME/HP, the vast majority of the cell population develops into terminally differentiated adipocytes. By use of these media and these special tissue culture manipulations, it is possible to obtain highly enriched populations of cells at each of the three states described above (35). These procedures were therefore used to prepare cell populations for the current studies.

It is important to emphasize that both nonterminally and terminally differentiated cells are adipocytes that are characterized by their expression of intracytoplasmic lipid droplets detected by phase contrast microscopy. This morphological criterion has been shown to correlate very well with enzymatic assays of adipocyte differentiation (9). The most significant difference between nonterminally and terminally differentiated adipocytes is the ability of the former to undergo DNA synthesis and clonal growth after restimulation with 30% FBS plus 50 μg/ml insulin in DME, whereas the latter are irreversibly growth arrested and are therefore refractory to growth factor restimulation.

Cell Culture: Normal/Human Keratinocytes

The procedure for preparing primary and secondary cultures of normal human keratinocytes was the same as that previously described in detail (41). Such cells are cultured in serum-free medium designated complete MCDB 153 prepared in our laboratory according to published procedures (2, 41). This medium also contains: 0.1 mM ethanolamine, 0.1 mM phosphoethanolamine, 5×10^{-7} M hydrocortisone, 5 μg/ml insulin, 10 ng/ml EGF, and 140 μg/ml bovine pituitary extract. Complete MCDB 153, containing 0.1 mM calcium, was used to prepare rapidly growing cultures of undifferentiated keratinocytes which do not express involucrin nor form morphological foci of stratified "crowns," which are excellent keratinocyte differentiation markers (31). To induce terminal differentiation of normal human keratinocytes, rapidly growing cells at a density of ~$5–8 \times 10^3$ cells/cm^2 were refed MCDB 153 containing all described additives and 2 mM calcium and were thereafter allowed to terminally differentiate at a higher cell density of ~2×10^4 cells/cm^2. In some studies, EGF and insulin were also removed from the medium, and/or 8 mM ethionine (Aldrich Chemical Co., Milwaukee, WI) was added to the medium to promote even more efficient keratinocyte growth arrest and differentiation (40). This was typically performed in lower density cultures. After incubation under these conditions for 7–12 d, maximum differentiation of 45–75% of the cells could typically be detected by use of a variety of keratinocyte differentiation markers including morphological, histochemical, and immunological techniques that have been previously described in detail (39–41).

mAbs

A series of six mAbs were used in these studies. Antibodies iD2 and fA12 are mouse IgM mAbs prepared against hnRNP core proteins (11). Antibodies AC88, 4F3, 7D11, and D7a are mouse IgG mAbs against hsp90; 4F3, 7D11, and D7A were prepared against chicken hsp90 and AC88 against an hsp90 homologue of the water mold *Achlya ambisexualis* (30). These antibodies were used for Western blotting at 1–10 μg/ml.

SDS-PAGE and Western Blotting

Samples for one-dimensional electrophoresis which were dissolved in the SDS sample buffer, were boiled for 2 min, and then resolved by electrophoresis on 7.5% acrylamide gels as described (10). Western blotting and antibody probing procedures were performed as described (12, 28). Briefly, after the transfer of proteins onto nitrocellulose sheets, incubation was carried out for 1 hour at room temperature in the Western buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% [vol/vol] Tween 20, and 0.1% [wt/vol] BSA) to block nonspecific binding of antibody to nitrocellulose. Probing was performed overnight at 4°C in the Western buffer containing 1–10 μg/ml of the mAbs. Nitrocellulose sheets were subsequently washed with Western buffer and probed for 2 h at room temperature using a second antibody, alkaline phosphatase–conjugated goat anti-mouse Ig antibody (Fischer Biotech, Orangeburg, NY). After extensive washing with Western buffer, the immunocomplexes on the nitrocellulose were visualized with 330 μg/ml nitroblue tetrazolium and 166 μg/ml 5-bromo-4-chloro-3-indolyl phosphate in 100 mM Tris-HCl, 100 mM NaCl, pH 9.5, containing 50 mM $MgCl_2$. In some experiments a radioactive secondary antibody was used to detect the immunocomplexes (12). For total protein visualization, nitrocellulose sheets were stained with 0.1% (vol/vol) India ink in PBS with 0.3% Tween 20 for 4–6 h.

Two-dimensional NEPHGE electrophoresis was performed by the O'Farrell method (18). The NEPHGE lysis buffer contained 9.5 M urea, 2% wt/vol Triton X100, 1.6% wt/vol ampholine 5-7, and 0.4% wt/vol ampholine 3.5-10 (Servalyte; Serva Fine Biochemicals, Garden City Park, NY) and 5% vol/vol 2-mercaptoethanol.

Proliferation Assays

To evaluate the proliferative potential of 3T3 T cells and human keratinocytes, two standard assays were used and both have been previously described in detail (9, 35). First, the ability of cells to incorporate [^{3}H]thymidine into DNA during the S phase of the cell cycle was evaluated using autoradiographic methods (6, 9). Second, the clonogenic potential of cells was assayed wherein ~500 cells were plated onto dishes in optimum growth medium, i.e., DME/30% FCS plus 50 μg/ml insulin or complete MCDB 153, respectively, and cultured therein for 10–14 d after which, the percentage of cells that formed colonies greater than four to eight cells was determined (35).

Preparation of Cell Extracts

Biochemical analyses were performed on total cell homogenates and on specific cell fractions. To prepare total homogenates, cells were scraped from dishes with a rubber policeman and washed twice with PBS, pH 7.4. Cells were lysed by addition of 50 μl of the SDS sample buffer (125 mM Tris-HCl, pH 6.8, 5% [vol/vol] 2-mercaptoethanol, 1% [wt/vol] SDS, 10% [vol/vol] glycerol, and 0.025% [wt/vol] bromophenol blue). Samples were mixed on a vortex apparatus and stored at −70°C until used for gel electrophoresis.

To prepare cytosol and nuclear fractions, a modification of a nuclear isolation method was used (12). Briefly, cells were harvested by scraping with a rubber policeman and were washed twice with PBS, pH 7.4. Cells were lysed by addition of a hypotonic solution containing 10 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 15 mM KCl, and 0.75% Triton X-100 (TKM buffer). Nuclei were sedimented over a layer of 1 M sucrose in TKM buffer. The remaining supernatant was mixed with an equal volume of 2× SDS sample buffer (see above) and used as the cytosolic fraction. The nuclear pellet was extracted with 0.35 M NaCl and 50 mM Tris, pH 7.4, and this extraction was also dissolved in 2× SDS sample buffer. Additional methods described below were used to isolate more pure nuclear preparations from various tissues and specimens.

Preparation of Nuclei from Cultured Cells

Intact cells were harvested and washed several times in ice cold solution of PBS and collected by centrifugation. The cell pellet was then lysed by addition of TKM containing 0.75% Triton X-100 and the lysate was centrifuged at 15,000 rpm for 10 min in a microfuge to sediment the nuclei. The nuclear pellet was washed once in TKM without Triton X-100 and directly lysed by addition of either SDS sample buffer (125 mM Tris, 1% SDS, 0.025% bromphenol blue, 10% glycerol, and 2.5% mercaptoethanol [pH 6.8]) and boiled, or the nuclear pellet was lysed in the two-dimensional nonequilibrium pH gradient gel electrophoresis (NEPGHE) sample buffer (described below) and stored at −70°C for subsequent analysis.

Table I. Characterization of Proliferative Potential of Native 3T3 T Cells and Transformed CSV3-1 Cells at Different Biological States and the Relative Abundance of P²Ps

Cell type	Biological state	Growth characteristic	Differentiation characteristic	Proliferative potential	Relative P²P abundance
3T3 T	Rapid growth state	Growing	Undifferentiated	+	+
	Serum deficiency state	Reversibly arrested	Undifferentiated	+	+
	Nonterminal differentiation	Reversibly arrested	Adipocyte	+	+
	Terminal differentiation	Irreversibly arrested	Adipocyte	−	−
CSV3-1*	Rapid growth state	Growing	Undifferentiated	+	+
	Nonterminal differentiation	Reversibly arrested	Adipocyte	+	+

* CSV3-1 cells do not efficiently undergo the terminal step in differentiation.

Isolation of hnRNP on Sucrose Gradients

The 30–40S subcomplexes of hnRNP were extracted and isolated by the method of Wilk et al. (37, 38). Typically, 1×10^7 cells were washed twice with PBS and resuspended in 10 mM Tris, 10 mM NaCl, and 1.5 mM $MgCl_2$, pH 7.0, at 10^6 cells/ml. The nonionic detergent NP40 was added to cells to a final concentration of 0.5%. After 10 min on ice, the cells were homogenized in a type B Dounce homogenizer. The homogenate was centrifuged for 10 min at 800 *g* and the supernatant removed. The pellet was washed sequentially with ice-cold 10 mM Tris, 100 mM NaCl, and 1.5 mM MgCl (STM, pH 7.0), and then with the same buffer at pH 8.0. The cells were resuspended in STM pH 8.0 at $3–5 \times 10^6$ cells/ml and stirred slowly for 30 min at 22°C. Insoluble material was sedimented by centrifugation at 800 *g* for 10 min and discarded. This procedure was then repeated. After sedimentation of insoluble material, the supernatants were pooled and applied to a 15–30% sucrose gradient in the extraction buffer. The gradient was centrifuged for 16 h at 90,000 *g* and then fractionated into 1-ml aliquots. Subsequent to determination of OD values by spectrophotometry (A260) in each sample, the total protein in each sample was precipitated by chloroform/methanol (33). Precipitated proteins in each sample were suspended in SDS sample buffer, boiled for 2 min and analyzed by PAGE and Western blotting.

For the identification of antigens associated with larger and more native hnRNP complexes, cell extracts were prepared in the presence of ribonuclease inhibitor. HeLa cell nuclei were isolated according to Martin and McCarthy (17) and washed twice with STM (pH 7.0) containing 100 U/ml placental ribonuclease inhibitor (Amersham Corp., Arlington Heights, IL). The washed nuclei were sonicated in 4 pellet volumes of STM pH 7.0 with RNase inhibitor and the debris was removed by centrifugation. The extract was resolved by sedimentation for 16 h at 50,000 *g* on a 35-ml 15–40% sucrose density gradient containing STM pH 7.0 (12). Absorbance at 254 nm was monitored using an UV analyzer and 3-ml aliquots were collected for the analysis of proteins by immunoblotting.

Results

The data in this manuscript will show that the abundance of a group of basic nuclear proteins, designated P²Ps for proliferation potential proteins, selectively and markedly decreases only in cells that have irreversibly lost their potential to proliferate in association with terminal differentiation. P²Ps were detected by Western blotting with antibodies against hnRNP core proteins or hsp90 and Table I summarizes the relationship between proliferation potential and the abundance of P²Ps.

To evaluate potential changes in the abundance of different antigens during the process of differentiation, a variety of mAbs, including those against hnRNP proteins (11) and hsp90 were used. One designated AC88 recognizes an epitope in hsp90 that is well conserved among eukaryotes (30). Western blots of total cellular extracts of undifferentiated 3T3 T

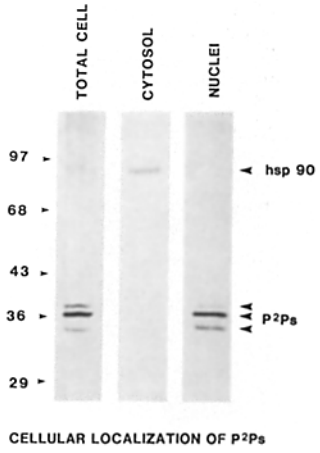

Figure 1. Localization of P²Ps to the nucleus in 3T3 T cells. Rapidly growing 3T3 T cells were either prepared as total cell lysates by homogenization in SDS sample buffer or fractionated into cytosolic and nuclear preparations as described in Materials and Methods. Samples were electrophoresed on 10% SDS-polyacrylamide gels and transferred to nitrocellulose by Western blotting. P²Ps and hsp90 were identified by probing the nitrocellulose with antibody AC88 and subsequent visualization of the protein bands by use of an alkaline phosphatase conjugated rabbit antimouse immunoglobulin. Each lane represents an approximately equal number of cells. Cytosol and nuclear fractions were derived from the same sample of cells. Protein molecular mass standards indicated by arrows on the left of the figure are phosphorylase b, 97 kD; BSA, 68 kD; ovalbumin, 43 kD; lactate dehydrogenase, 36 kD; and carbonic anhydrase, 29 kD.

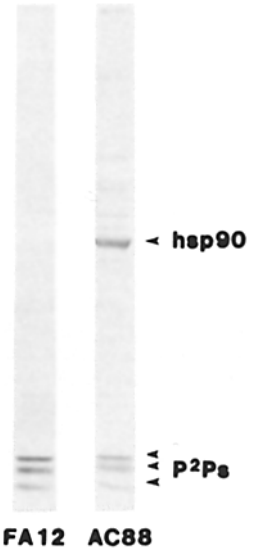

Figure 2. A comparison of the antigens recognized by antibodies AC88 and fA12 in whole 3T3 T cell extracts shows that AC88 recognizes both P²Ps and hsp90 but that fA12 only recognizes P²Ps.

cells were probed to determine if AC88 recognized hsp90 in these cells. The results showed that AC88 not only recognized hsp90 but, in addition, a group of distinct proteins designated P²Ps with 30–40-kD apparent molecular masses.

Fig. 1 shows that P²Ps have a nuclear localization in 3T3 T cells, in contrast to hsp90, which is primarily localized to the cytoplasm. Detection of P²Ps is specifically dependent on the presence of AC88 or fA12 antibodies since omission of these antibodies resulted in the failure to detect P²Ps by Western blotting (data not shown). In addition, presaturation of antibody AC88 with purified hsp90 eliminated the Western blot signal for both hsp90 and for the 30–40-kD P²P proteins.

To determine if other anti-hsp90 antibodies could recognize P²Ps, three additional hsp90 mAbs, designated 4F3, D7a, and 7D11, were incubated with Western blots of cellular extracts from the chick oviduct because these antibodies are specific for avian hsp90 (30). Preliminary experiments showed that the results in Fig. 1 could be duplicated using chicken tissue. Similar results have been observed with the water mold *Achlya*, rabbit tissues, and human tissues (data not shown). These antigens therefore appear to be very highly conserved. In chickens, all four hsp90 antibodies recognized hsp90, but P²Ps were only detected by antibodies AC88 and 4F3 (see Materials and Methods) (30). The latter antibody gave a relatively weak, but reproducible reaction. The absence of reactivity with P²Ps using avian hsp90-specific mAbs D7a and 7D11 demonstrates specificity of 4F3 and AC88 for P²P antigens and rules out the possibility of a general affinity for nonspecific antibodies to interact with P²Ps. Based on these results it was concluded that P²Ps and hsp90 share at least one epitope in common.

The possibility that P²P antigens might result from the artifactual proteolysis of hsp90 was explored even though several biochemical characteristics of P²Ps and hsp90 argue against this possibility. For example, hsp90 is an acidic cytosolic protein with an isoelectric point reported to be between 4.5 and 5.5 (32). In contrast, P²Ps are basic nuclear proteins with pIs of ~9.0 (see Fig. 4). A series of experiments using protease inhibitors showed no effect on P²P or hsp90 abundance suggesting that P²Ps are not artifactual in vitro proteolytic products of hsp90. More important, P²Ps were also recognized by the fA12 mAb prepared against core proteins of hnRNP particles and this antibody does not recognize hsp90 epitopes. Fig. 2 demonstrates this finding.

The testing of antibodies against hnRNP proteins was suggested by preliminary efforts to fractionate P²Ps. The P²Ps appeared to be associated with larger complexes and espe-

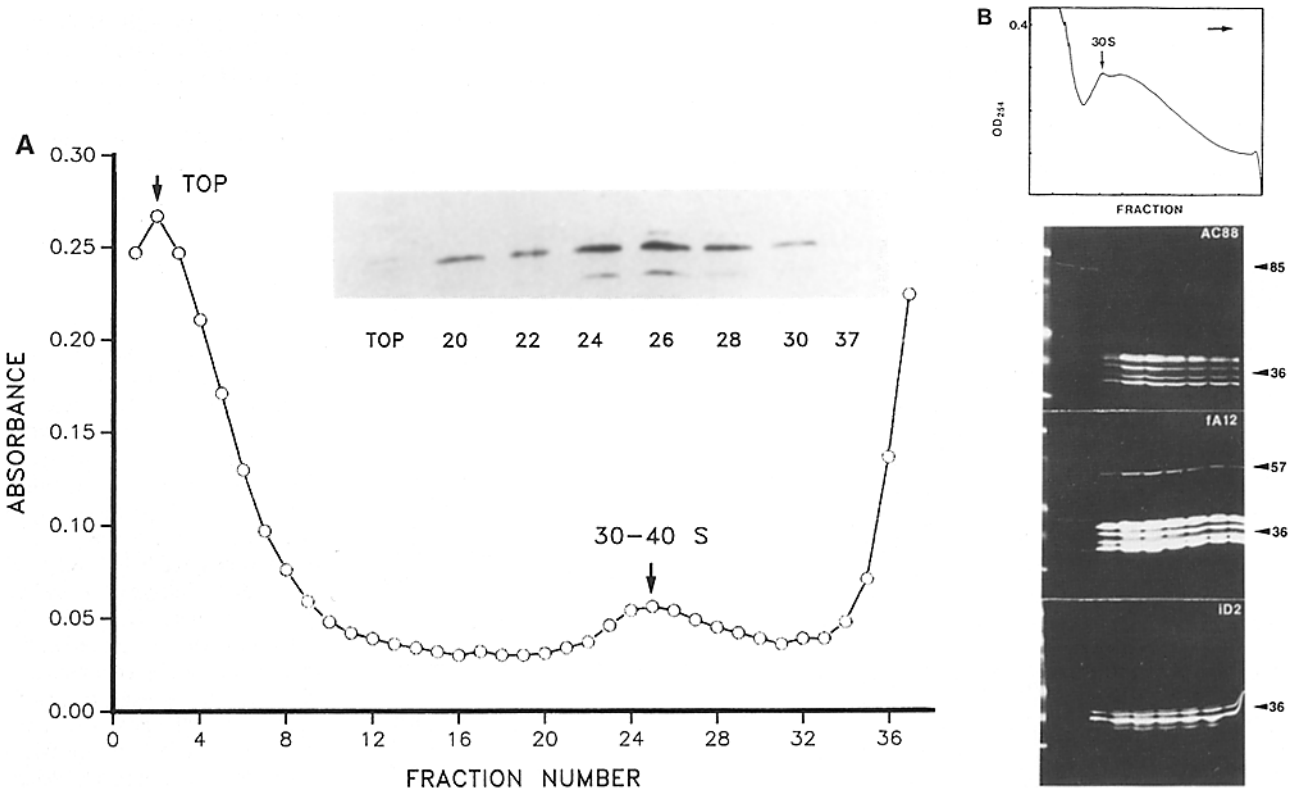

Figure 3. (*A*) P²Ps are found within the 30–40S peak of nuclear extracts on sucrose density gradients. Nuclear extracts from 3T3 T cells were centrifuged through a 15–30% sucrose gradient as described in the Materials and Methods. Fractions collected across the gradient were resolved by one-dimensional gel electrophoresis on 10% polyacrylamide gels and Western blotted with AC88 antibody. The profile of the absorbance at 260 nm is shown; *inset* illustrates selected fractions from the Western blot detection of P²Ps. (*B*) P²Ps cosediment with known core proteins in large hnRNP complexes prepared in the presence of placental RNase inhibitor. HeLa cell nuclear extracts were resolved by centrifugation on 15–40% sucrose gradients. Complexes detected on this gradient range from the 30S subcomplex to structures of ~200S. The proteins contained in gradient fractions were electrophoresed, Western blotted, and probed with mAbs using the radioactive second antibody method. The hnRNP core proteins and the AC88 antigen cosediment precisely throughout the gradient. Note that the fA12 antibody also reacts with an hnRNP protein of 57 kD, not recognized by AC88.

cially hnRNPs which represent a major particulate entity within nuclei (5, 13). Based on these observations, the possibility was explored that P²Ps might be related or identical to protein moieties of hnRNP complexes (13, 15, 16). When nuclei are extracted without extensive precautions to prevent ribonuclease action, hnRNP complexes are commonly isolated as relatively homogeneous 30–40S subcomplexes. We therefore examined the distribution of P²Ps in conventional extracts of hnRNPs resolved by sucrose gradient sedimentation (37, 38). Fractions collected after centrifugation were analyzed for the presence of P²Ps by Western blotting using AC88 antibody. The data show the cosedimentation of the P²Ps with the optical density peak of the 30–40s hnRNP subcomplex (Fig. 3 *A*); there are no significant amounts of the 30–40-kD antigen at the top or bottom of the gradient.

Because these preliminary findings suggested an association of P²Ps with hnRNP complexes, we sought to determine if the proteins were also present in large, presumably more native complexes. A nuclear extract from HeLa cells prepared in the presence of placental ribonuclease inhibitor was resolved by sucrose gradient sedimentation. Complexes of 30–200S that contained hnRNP core proteins recognized by the mAbs iD2 and fA12 were obtained (Fig. 3 *B*). The 30–40-kD antigens detected by AC88 cosedimented precisely with the bona fide hnRNP polypeptides. Extremely mild RNase treatment of extract converted the bulk of all three antigens to a form that sedimented at 30S (results not shown). Thus, the P²P proteins appear to be constituents of native hnRNP complexes, and behave as if they were novel members of the basic core protein family.

P²Ps Represent Novel Components of hnRNP

To further characterize the relationship between hnRNP particles and P²Ps, the properties of P²P antigens were compared with those of previously described major core polypeptides of hnRNP complexes which fall into three distinct size groups termed A, B, and C (13). Based on their molecular size, P²Ps could represent group A or group B hnRNP proteins because these polypeptides are basic in charge and range in size between 35 and 40 kD. However, the A, B, and C groups each contain multiple proteins and the total number of hnRNP proteins has been estimated to be >20 (5, 12, 37). To determine whether P²Ps are similar or identical to such hnRNP proteins, we compared, by two-dimensional NEPHGE gel electrophoresis and Western blotting, the pattern of P²Ps with those of previously described hnRNP proteins. This analysis was performed on HeLa cell extracts to allow better comparisons with previous reports on HeLa hnRNP proteins (12, 37). Fig. 4 shows that antibody iD2 indeed recognizes several major hnRNP proteins in the A and B groups and presents the typical arrowhead pattern (12). In contrast, fA12 recognizes a group of hnRNP proteins that are mostly different from those recognized by iD2, although there are possible overlapping spots recognized by both antibodies. The pattern of staining with AC88 closely resembles that obtained with fA12 for P²P associated hnRNPs. To identify regions of coincidence, combinations of these antibodies were used next. Probing with AC88 and fA12 in combination consistently demonstrated that AC88 epitopes are a subset of protein spots recognized by fA12 alone. Although a small number of iD2-recognized protein spots are coincident with those recognized by fA12, iD2, and AC88, epitopes do not appear to exhibit detectable overlaps. The AC88 antigens appear to be among the C1, B1, and B2 hnRNP proteins described by Wilk et al. (37) or the B1 and B2 proteins described by Dreyfuss et al. (5).

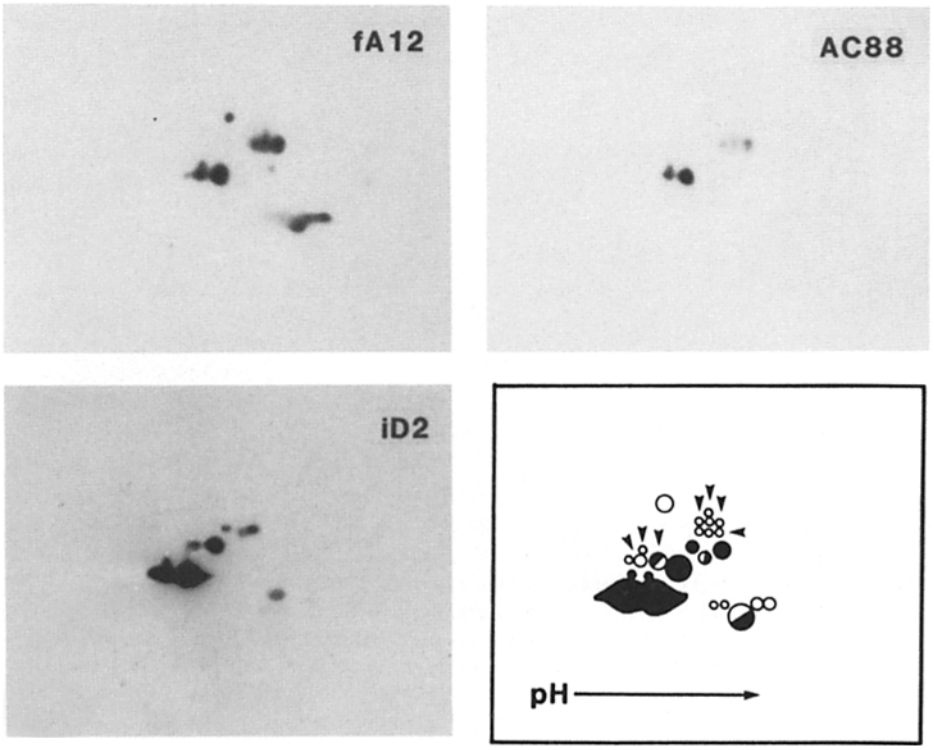

Figure 4. Comparison of P²Ps and known hnRNP proteins on two-dimensional NEPHGE gels. To determine whether P²Ps are identical or similar to the previously described major core polypeptides of hnRNP particles, equal quantities of isolated HeLa cell nuclei were lysed in NEPHGE lysis buffer and electrophoresed on two-dimensional NEPHGE gels. After Western blotting, one of the three antibodies or a combination of antibodies was used to identify antigens. Each panel illustrates the antigens recognized on Western blots by the antibody designated in each case. The relationship of these antigens is indicated by a schematic derived by testing combinations of the three antibodies. ○, antigens detectable by fa12; ●, antigens recognized by iD2 alone; and ◐, iD2, fA12 cross-reactive antigens. The arrows indicate the P²P antigens.

Regulation of P²Ps during Terminal Differentiation in 3T3 T Cells

Mesenchymal stem cells in culture regulate their proliferation and differentiation in a coordinated fashion. A model that describes linkages in the control of proliferation and differentiation in these cells has been described by Scott et al. (22, 23). Those data show that 3T3 T cells can develop into either nonterminally or terminally differentiated adipocytes. Both types of adipocytes represent mature fat cells on the basis of morphology; however, nonterminal adipocytes retain their proliferative potential, whereas terminal adipocytes do not. That is, terminally differentiated adipocytes cannot be stimulated to proliferate by any known growth factors or mitogens (35). Table I summarizes these important biological characteristics and stresses that only terminally differentiated 3T3 T adipocytes lose their proliferative potential and show a marked decrease in P²P abundance.

The existence of separate states of nonterminal and terminal differentiation makes it possible to distinguish events related to differentiation per se from those that are selectively associated with the irreversible loss of proliferative potential which occurs only with terminal differentiation. Therefore, to determine if changes in the abundance of P²Ps occurs only with terminal differentiation, equal numbers of 3T3 T cells at different biological states were isolated and cells were processed for Western blotting; and P²Ps were identified by use of the AC88 antibody. Similar results were obtained with the fA12 antibody (data not shown). The results (Fig. 5) show a strong correlation between the loss of proliferative potential and the decreased abundance of P²Ps. It is important to stress that no major change in hsp90 abundance which served as an internal control was detected during this interval. Fig. 5 *B* also shows that during the process of differentiation-induced irreversible loss of proliferative potential, only slight changes occur in the majority of core hnRNP proteins that are recognized by the iD2 antibody probe. In this regard, maximum nonterminal adipocyte differentiation occurs between days 5 and 7, when ~30–80% of cells were differentiated but retained their proliferative potential. Fig. 5 *A* shows no major change in P²Ps during this interval. In contrast, from days 7 to 12, the relative percentage of terminally vs. nonterminally differentiated cells progressively increases concurrent with loss of proliferative potential.

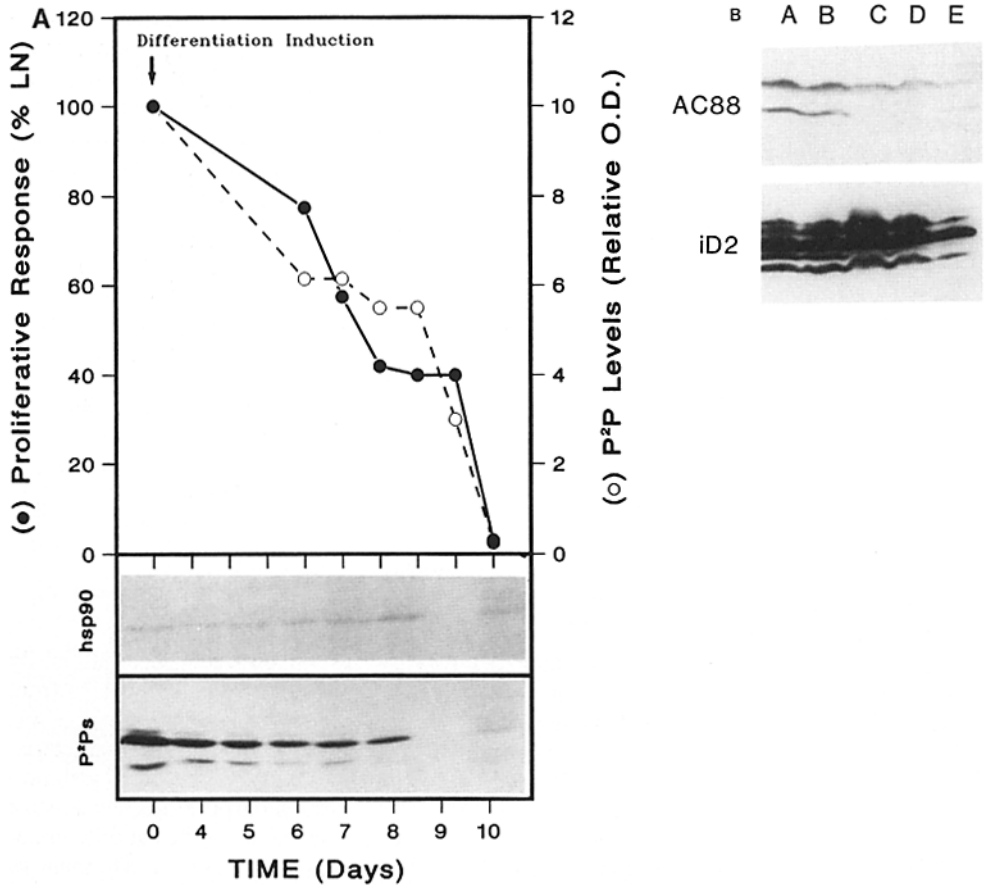

Figure 5. (*A*) Kinetics of the reduction in steady-state levels of P²Ps associated with the loss of proliferative potential that occurs during the terminal differentiation of 3T3 T cells. Low-density, rapidly growing 3T3 T cells were induced to undergo terminal differentiation in heparinized DME containing 25% human plasma as described in Materials and Methods. Duplicates of samples on the days designated were removed and analyzed. One sample was used to identify the steady state levels of P²Ps and hsp90 by probing Western blots of total cellular proteins with mAb AC88. In these assays, equal numbers of cells were evaluated. Relative optical density of P²Ps in each sample was subsequently determined by densitometric scanning of Western blots. A duplicate sample was also used to determine the proliferative potential of cells at different differentiation states. More specifically, the proliferative response of differentiating cells was determined by treating them with high growth factor concentrations and measuring [³H]thymidine incorporation into DNA as described in Materials and Methods. (*B*) Comparison between iD2 and AC88 epitopes detected during the differentiation of 3T3 T cells in vitro. Equal numbers of cells on specific days during differentiation were collected and lysed in the SDS lysis buffer. Each sample was then divided in half and electrophoresed on 12% polyacrylamide gels, Western blotted, and probed with either iD2 or AC88 separated. (*A*) Cells before induction of differentiation; (*B–E*) cells on different days after differentiation induction; (*B*) day 2; (*C*) day 5; (*D*) day 8; and (*E*) day 12. During the differentiation response cells first become nonterminally differentiated at approximately day 5. Then some cells begin to undergo the terminal event in differentiation between days 6–10. Finally, by approximately day 12, most of the adipocytes have lost their proliferative potential and are therefore terminally differentiated.

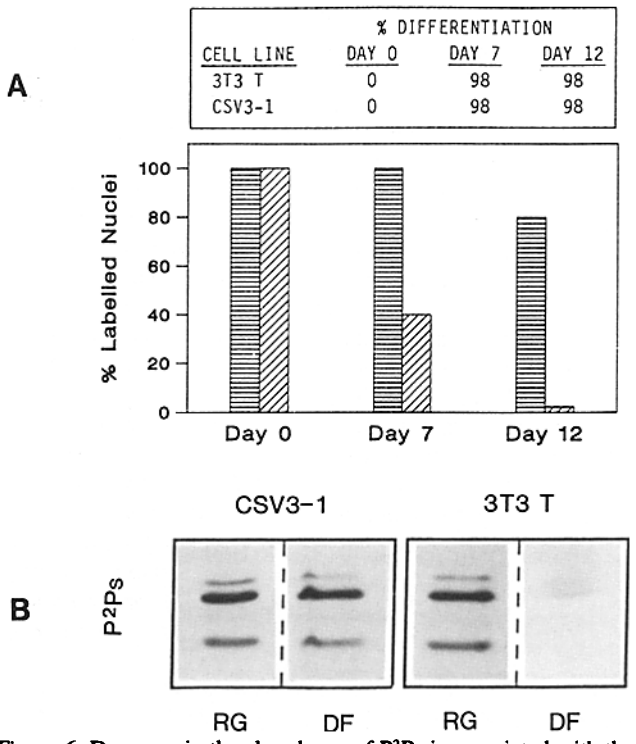

Figure 6. Decrease in the abundance of P²Ps is associated with the loss of proliferative potential that occurs in association with terminal differentiation in nontransformed 3T3 T cells. In contrast, in a SV40-transformed 3T3 T clone designated CSV3-1 that can differentiate but not terminally, no change in P²P levels occurs. *A* shows that 3T3 T and CSV3-1 cells differentiate equally well. However, only 3T3 T cells lose their ability to be restimulated to grow as the differentiation process proceeds, i.e., days 0, 7, and 12. These data were derived by autoradiographic analysis of [³H]thymidine incorporation and are presented as percent labeled nuclei. Data on CSV3-1 are on the left bar graphs with parallel lines; 3T3 T data on the right bar graphs with diagonal lines. *B* compares the relative abundance of P²Ps in rapidly growing (*RG*, day 0) and differentiated (*DF*, day 12) 3T3 T and CSV3-1 cells by use of Western blotting using the AC88 antibody and shows that the abundance of P²Ps only decreases in adipocytes that irreversibly lose their proliferative potential.

During this latter interval, the level of P²Ps progressively decreases even though the percentage of adipocytes remains constant.

Additional assays were performed to further evaluate whether the decrease in the abundance of P²Ps is directly associated with the terminal event in differentiation or differentiation per se. For this purpose, we evaluated whether the abundance of P²Ps is reduced during differentiation of a unique clone of SV40-transformed 3T3 T stem cells, designated CSV3-1 (21). This clone retains the ability to differentiate to an extent comparable to parental 3T3 T cells, but cannot undergo extensive terminal differentiation (Table I). Fig. 6 *A* shows that both 3T3 T and CSV3-1 cells differentiate equally well but that only 3T3 T adipocytes undergo terminal differentiation. Furthermore, as adipocyte differentiation occurs, 3T3 T cells lose their ability to be stimulated to grow whereas CSV3-1 adipocytes do not lose their proliferative potential. Fig. 6 *B* shows Western blot analysis of P²Ps in rapidly growing and differentiated 3T3 T and CSV3-1 cells. In contrast to normal 3T3 T stem cells, which show a marked decrease in P²P levels during terminal differentiation, no detectable change in the abundance of P²Ps in CSV3-1 cells is observed, even when such cells are cultured for extended intervals in medium that promotes terminal differentiation in parental 3T3 T cells. Therefore, it appears that if cells fail to undergo terminal differentiation, the decrease in abundance of P²Ps does not occur. These data further support the conclusion that reduction in abundance of P²Ps is not associated with differentiation per se but rather with the terminal event in differentiation.

Reversible Growth Arrest in 3T3 T Stem Cells by Serum Deprivation Does Not Modulate P²P Levels

The above data demonstrate a strong correlation between the irreversible loss of proliferative potential in terminally differentiated cells and the marked decrease in the abundance of P²Ps. To further substantiate this observation, we examined the steady-state levels of P²Ps at a reversible growth arrested state induced by serum deficiency in 3T3 T cells (Table I). Such reversibly growth-arrested cells retain their proliferative potential because they can be subsequently restimulated to reinitiate cell cycle progression and cell division. We also evaluated whether the abundance of P²Ps changed after reversibly growth-arrested cells are stimulated to reinitiate proliferation. For this purpose, the growth of 3T3 T stem cells was arrested by culture in serum-depleted medium and the abundance of P²Ps was assayed by immunodetection of Western blots. Similarly, after growth was arrested which was determined by a markedly decreased [³H]thymidine incorporation into DNA and by the absence of increases in cell density, specimens were sequentially isolated after growth restimulation with medium containing 10 or 30% FBS to evaluate P²P levels. Fig. 7 shows that no significant change in the abundance of P²Ps occurred during reversible growth arrest nor during the reinitiation of proliferation in quiescent cells. These data demonstrate that P²P levels are not affected by reversible growth arrest nor by serum-induced growth stimulation in 3T3 T stem cells. More importantly, these data suggest that unlike serum/growth factor responsive proteins (1, 14, 20), the decline in P²P levels is a specific event occurring only in conjunction with loss of proliferative potential during terminal differentiation and not with reversible quiescence.

Modulation of P²Ps during Differentiation of Normal Human Keratinocytes

Preliminary results suggested that P²Ps are evolutionarily well conserved in a wide range of eukaryotic species from filamentous fungi to human cells (data not shown). It was therefore possible to evaluate whether or not the characteristics of P²Ps change during the process of terminal differentiation of other cell types. For this purpose, the steady-state levels of P²Ps were analyzed during the terminal differentiation of normal human keratinocytes. The development of serum-free media for the culture of normal human skin epithelial cells has facilitated the study of the biological mechanisms that regulate keratinocyte proliferation and differentiation (2). Wille et al. have established that, as in 3T3 T stem cells, the proliferation and differentiation of human keratinocytes are integrally regulated (41). In contrast to 3T3 T cells, however, the process of differentiation in normal human ker-

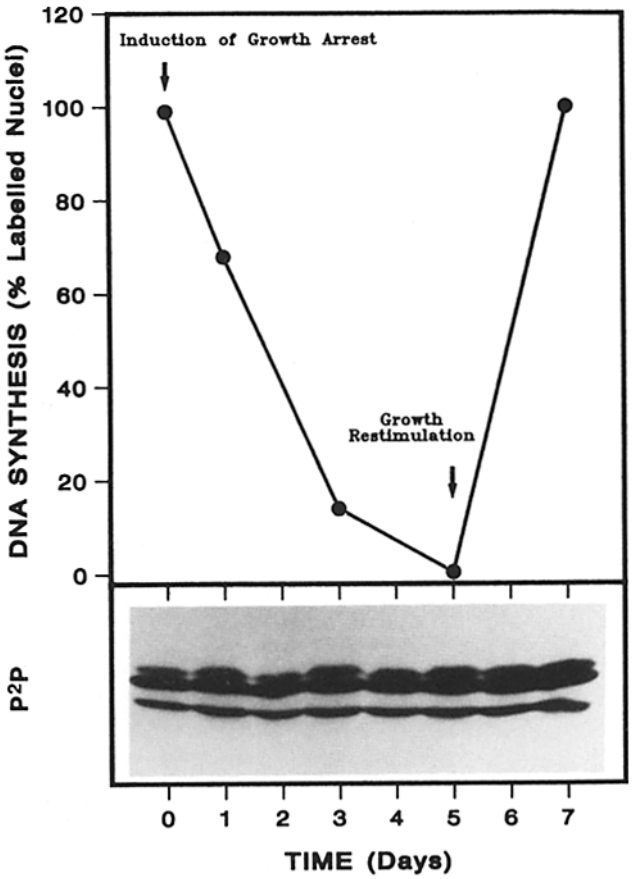

Figure 7. Reversible arrest of 3T3 T cell growth by serum deprivation does not alter P²P levels. Duplicate samples of equal numbers of 3T3 T cells were cultured in medium containing 0.5% FBS to induce reversible growth arrest. On each day thereafter, one sample of each duplicate set of specimens was analyzed by Western blotting of total cellular proteins. P²Ps were identified by probing with mAb AC88. The other sample was used for determination of percentage of cells undergoing DNA synthesis by measurement of [³H]thymidine incorporation by autoradiography. After growth arrest occurred by day 5, cells were stimulated to grow by the addition of 30% FBS and were subsequently analyzed on days 6 and 7.

atinocytes is not as highly synchronized. For example, when keratinocytes are placed in differentiation-promoting medium, different members of the population growth arrest and differentiate with distinct kinetics. Some differentiate within 2–4 d, whereas others may require 6–10 d to differentiate. Indeed, some normal human keratinocytes never fully differentiate in vitro even though they can all eventually lose their proliferative potential. Nonetheless, a good correlation does exist between the expression of differentiation markers and loss of colony-forming efficiency in normal human keratinocytes (36).

Cultures of normal human keratinocytes were, therefore, induced to undergo terminal differentiation in vitro, during which time cell samples were examined for their differentiation characteristics and for loss of proliferative potential by assaying their clonogenic potential, i.e., the ability of single cells to form colonies. Samples containing equal numbers of cells were also evaluated by Western blot analysis to assay P²P levels. Total cellular protein from each sample was analyzed by SDS-PAGE, blotted onto nitrocellulose, and probed with AC88 antibody. Fig. 8 shows that in normal human keratinocytes, the abundance of P²P is also selectively reduced when cells irreversibly lose their proliferative potential in association with terminal differentiation. Additional data not plotted show the extent of keratinocyte differentiation, as determined by involucrin expression and cell stratification, which are excellent markers of keratinocyte differentiation: day 0, 0%; day 3, 15%; day 6, 30%; and day 9, 45%. This demonstrates that in two mammalian species and two separate cell types, downregulation of P²P levels correlates closely with the loss of proliferative potential associated with terminal differentiation.

Discussion

Delineation of the mechanisms that regulate proliferation and differentiation of mammalian cells is essential to understand a variety of health-related problems, including carcinogenesis. One very important question concerns how cells regulate the process of terminal differentiation wherein they irreversibly lose their proliferative potential. In previous studies, the biological events in the terminal differentiation of 3T3 T mesenchymal stem cells (23, 34–36) and normal human keratinocytes (39–41) were established. In these studies, both cell systems were used to evaluate the steady-state levels of a group of distinct nuclear proteins, designated P²Ps for proliferation potential proteins. These antigens were identified by virtue of their strong cross reactivity with mAbs to hnRNP proteins that share an epitope with hsp90.

The most important observation in this study is that P²P

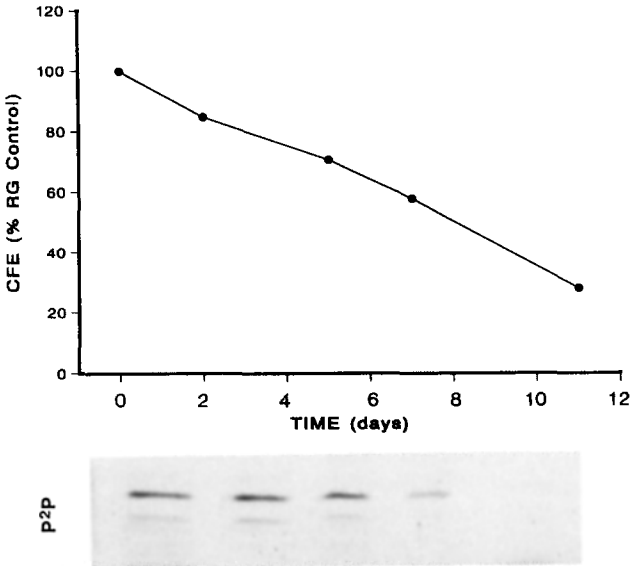

Figure 8. Reduction in the steady-state level of P²Ps during the terminal differentiation of normal human keratinocytes. Normal diploid human keratinocytes were induced to undergo terminal differentiation by culture in serum-free medium containing 2 mM Ca^{2+}. Samples of keratinocytes undergoing terminal differentiation were then periodically collected and analyzed for P²P levels by Western blotting of total cellular proteins using antibody AC88. Duplicate samples of keratinocytes were also used to assay the extent of differentiation and cellular proliferation potential by the colony-forming efficiency method described.

abundance markedly decreased in association with the loss of proliferative potential during terminal differentiation in two independent mammalian cell types. To establish that this decrease in the abundance of P²Ps occurs specifically during the terminal event of differentiation a temporal comparison was made between the loss of P²Ps and the loss of responsiveness to mitogenic stimulation during differentiation of 3T3 T cells. The results demonstrate a strong correlation between decreased steady-state levels of P²Ps and the percentage of irreversibly terminally differentiated cells. Second, the abundance of P²Ps does not change when murine 3T3 T stem cells were induced to reversibly growth arrest by serum deprivation nor was there any significant change in the level of these proteins when such growth-arrested cells were restimulated to proliferate. Third, it was shown that in CSV3-1 cells, an SV40-transformed cell line that undergoes nonterminal differentiation but not terminal differentiation, changes in the abundance of P²Ps do not occur. This strongly suggests that changes in P²P levels are not a consequence of differentiation per se, but rather suggests that these changes are specifically associated with the irreversible loss of proliferative potential during terminal differentiation. These observations are most significant because very few if any specific changes in other nuclear proteins have been reported in association with the terminal event in differentiation (25, 27).

Another important observation is that P²Ps represent a subset of hnRNP proteins. This observation raises the possibility that posttranscriptional regulation of gene expression via changes in specific hnRNP components may play a significant role in the control of a cell's proliferative potential. HnRNP proteins have long been presumed to mediate the correct packaging of hnRNA within the nucleus much in the same way as nucleosomal proteins package DNA into chromatin (16). Recent evidence further suggests that hnRNP-associated proteins may be essential components of pre-mRNA splicing and processing machineries in the nucleus. For example, it has been shown that the class C hnRNP proteins are involved in splicing (3, 5). The abundance of other core polypeptides of hnRNP proteins, i.e., the complement of groups A and B, has also been demonstrated to change during the cell cycle (12). Finally, electron microscopic observations of chromosome preparations suggest that specific pre-mRNAs very rapidly become associated with snRNP and hnRNP protein particles (7, 19). Recent findings that some hnRNP proteins exhibit RNA binding specificities (29) support the possibility that different pre-mRNAs may require specific combinations of hnRNP and snRNP proteins for their correct splicing, processing, and nucleocytoplasmic transport. Data derived from these studies raise the interesting possibility that certain subsets of hnRNA binding proteins, i.e., those containing P²Ps, might be essential for the maturation of specific proliferation-associated pre-mRNAs.

The types of mRNA that may be dependent on P²Ps for their processing, maturation, and nucleocytoplasmic transport need to be further investigated by the use of more specific mAb probes with which it would be possible to immunoprecipitate and isolate hnRNA-protein complexes. Development of cDNA clones and subsequent derivation of mAbs to synthetic peptides should greatly facilitate this type of analysis. Because P²Ps share at least one epitope in common with eukaryotic hsp90, it will also be important to determine if hsp90 and P²Ps share any functional relationships. Finally, because defects in the control of terminal differentiation is associated with the process of carcinogenesis (24, 42), cancer cells need to be studied to determine the extent of aberrant P²P characteristics. For example, studies are now justified to determine if alterations in the biosynthesis and/or structure of P²Ps in cancer cells might contribute to abnormalities in their growth and differentiation characteristics.

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