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Review

Involvement of non-receptor protein tyrosine kinases in expression of differentiated phenotype by cells of retinal origin

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Introduction

Phenotypic expression of epithelia *in vitro* is affected by the status of their adhesiveness. This is, in turn, regulated by soluble factors, such as hormones and growth factors, and attachment factors, such as extracellular matrix (ECM) proteins. In a differentiating (or transdifferentiating) cell system, after the decision to establish (or to switch), the genomic program has been taken, attachment factors may further control its realization. Cells recognize proteins of the ECM by cell surface receptors, members of the integrin superfamily (Hynes, 1992), which often localize to strong cell-substratum adhesions known as focal contacts. Focal contacts (Fig. 1) are specialized membrane-cytoskeleton complexes associated with the ends of microfilament bundles and contain a set of focal contact-specific proteins such as vinculin, talin, tensin, paxillin and several others (Burridge *et al.*, 1988). Some of these proteins, e.g., vinculin and paxillin, are targets of tyrosine (Tyr) kinases (Sefton and Hunter, 1981; Turner *et al.*, 1990; Burridge *et al.*, 1992) and, in fact, focal contacts appear to be regulated by protein phosphorylation on Tyr (Rohrschneider *et al.*, 1982; Kellie, 1988; Kornberg and Juliano, 1992). Focal contacts by linking the cytoskeleton to proteins of the ECM (Singer, 1979; Hynes *et al.*, 1982; Singer *et al.*, 1984; Burridge and Fath, 1989) *via* the integrins (Hynes, 1992; Reichardt and Tomaselli, 1991; Ruoslahti, 1991), act as mechanical integrators of the intra- and extracellular environment (Opas, 1987; Ingber *et al.*, 1994).

The phenotypic expression of epithelial cells *in vitro* is adhesion-dependent in that development of strong cell-substratum adhesion favors the loss of differentiated traits while strong cell-cell adhesion favors expression of the differentiated phenotype. Strong intercellular adhesions are mediated in epithelial cells by tight junctions and zonulae adherens. Zonulae adherens associate with circumferential rings of microfilaments which actively contract and maintain the epithelial cell sheet under tension (Owaribe *et al.*, 1981; Owaribe and Masuda, 1982). Hence, zonulae adherens have been postulated to act as mechanical equivalents of focal contacts (Opas, 1987). At the molecular level, zonulae adherens and focal contacts share some components (e.g., vinculin), but also have components unique for each adhesion type (Geiger *et al.*, 1985, 1987). Thus, the cytoplasmic protein talin (Burridge and Feramisco, 1980) and several transmembrane integrins (Damsky *et al.*, 1985; Horwitz *et al.*, 1985; Tamkun *et al.*, 1986; Buck and Horwitz, 1987) may be considered molecular markers for focal contacts, while the cytoplasmic proteins, plakoglobin (Franke *et al.*, 1987) and catenins (Kemler, 1992; Piepenhagen and Nelson, 1993; Stappert and Kemler, 1993), and transmembrane cadherins (Hirano *et al.*, 1987; Takeichi, 1990; Tsukita *et al.*, 1992) may be considered molecular markers for zonulae adherens. In epithelial cells, the proteins of zonulae adherens are major targets of protein Tyr kinases (Maher *et al.*, 1985; Tsukita *et al.*, 1991; Volberg *et al.*, 1991, 1992; Matsuyoshi *et al.*, 1992). The products of c-*src* and c-*yes* are concentrated in the adherens' junctions (Tsukita *et al.*, 1991) and the junctions have elevated levels of Tyr-phosphoproteins compared to non-junctional areas (Shriver and Rohrschneider, 1981; Maher *et al.*, 1985). The level of Tyr phosphorylation in adherens junctions increases after inhibition of protein-Tyr phosphatases with sodium orthovanadate (Hecht and Zick, 1992), suggesting a role of Tyr phosphatases in their function (Tsukita *et al.*, 1991; Volberg *et al.*, 1991, 1992). Phosphorylation reciprocally modulates the stability of the two types of adherens junctions. Increased phosphorylation degrades the cell-cell junctions, while dephosphorylation stabilizes them (or restores previously degraded junctions). The opposite seems to be true for focal contacts (Volberg *et al.*, 1991, 1992). Because of the importance of adhesion in epithelial cell function, and of the role of Tyr phosphorylation in junctional stability, non-receptor Tyr kinases have been implicated in the regulation of expression of the differentiated epithelial phenotype (Ellis *et al.*, 1987; Vardimon *et al.*, 1991; Schmidt *et al.*, 1992; Zhao *et al.*, 1992).

Signal transduction and tyrosine kinases

The family of protein Tyr kinases can be divided into two groups: receptors and non-receptors (Hunter, 1987). As the receptor Tyr kinases have been a subject of numerous reviews (Hunter, 1987; Druker *et al.*, 1989; Sigal and Gibbs, 1989; Ullrich and Schlessinger, 1990; Schlessinger and Ullrich, 1992; Schmidt *et al.*, 1992), here we will concentrate only on the non-receptor type of protein Tyr kinases. The three major families of non-receptor Tyr kinases identified so far in normal cells are *src*, *abl* and *fps* gene families (Hunter, 1987; Kellie *et al.*, 1991; Brickell, 1992). We will further limit our considerations to two adhesion-related kinases, $pp60^{src}$ and a novel, structurally distinct non-receptor protein Tyr kinase, $pp125^{FAK}$ (Zachary and Rozengurt, 1992; Schaller and Parsons, 1993).

$pp60^{c\text{-}src}$ and $pp60^{v\text{-}src}$

The c-*src* gene is a prototype for several closely related cellular genes that encode Tyr kinases and share a common amino acid domain structure. The proteins of the src family contain a conserved region with a catalytic domain of approximately 260 amino acids which alone is sufficient for Tyr kinase activity. The catalytic domain contains sequences involved in ATP binding and also includes two Tyr residues which are themselves major targets for phosphorylation. Phosphorylation of the src protein inhibits its Tyr kinase activity while dephosphorylation causes the reverse effect.

The region of homology between the products of the c-*src* gene family also contains two conserved non-catalytic domains, SH_2 and SH_3 (*src* Homology Regions 2 and 3). Proteins that contain the SH_2 domain can interact with activated growth factor receptors, oncogene products and other Tyr-phosphorylated proteins (Glenney, 1992; Schlessinger and Ullrich, 1992). While SH_2 and SH_3 domains are involved in the regulation of src kinase activity

Abbreviations used in this paper: bFGF, basic fibroblast growth factor; BM, basement membrane; ECM, extracellular matrix; N-CAM, neural cell adhesion molecule; NR, neural retina; P-Tyr, phosphorylated tyrosine; RPE, retinal pigment epithelium; Tyr, tyrosine.

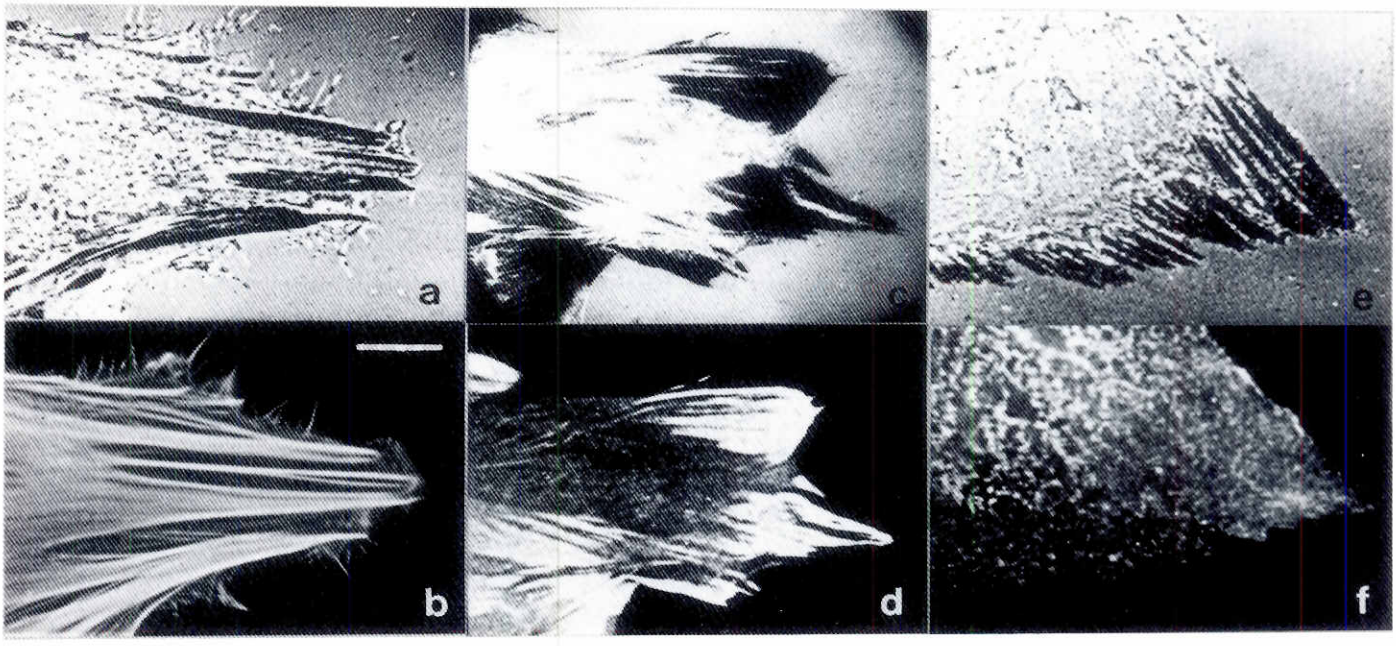

Fig. 1. Interference reflection microscopy (a,c,e) and fluorescence microscopy (b,d,f) of RPE cells showing patterns of cell-substratum adhesions in relation to the distribution of actin-containing microfilaments (b) vinculin (d) and spectrin (f) in the same cells. *For fluorescence microscopy paraformaldehyde-fixed and Triton X-100-permeabilized cells were incubated either with anti-vinculin or anti-spectrin primary antibodies or with FITC-conjugated actin-specific probe, phalloidin. Antibody binding was detected with FITC-conjugated secondary antibodies. Interference reflection microscopy visualizes areas of the most intimate cell contact with the substratum (the focal contacts) as black patches clearly discernible in* **(a,c,e)**. *While many actin-binding cortical proteins, e.g., vinculin* **(d)**, *are found in the focal contacts, other actin-binding cortical proteins, such as spectrin* **(f)**, *are not and display distribution that is entirely unrelated to the pattern of focal contacts* **(e)**. *Bar, 10 μm.*

(Pawson, 1988; Van Etten *et al.*, 1989; Cooper and Howell, 1993), there is evidence that the SH_3 domain is responsible for targeting of SH_3-containing proteins to their specific cellular locations (Bar-Sagi *et al.*, 1993). The amino acid sequences of the viral oncogene product $pp60^{v\text{-}src}$ and its normal cellular counterpart $pp60^{c\text{-}src}$ differ to a degree. 19 amino acids at the C-terminal end of the cellular *src* are replaced by 12 unrelated amino acids in the viral protein. As a result of this replacement a Tyr at position 527 is lost. Because phosphorylation of Tyr 527 reduces the kinase activity of c-*src*, the viral protein is constitutively more active. The $pp60^{c\text{-}src}$ may also be a target for other cellular protein Tyr kinases or a substrate for the widely expressed receptor-like protein Tyr phosphatase, PTPα (Hunter, 1989; Okada and Nakagawa, 1989; Zheng *et al.*, 1992). Finally, the regulation of $pp60^{c\text{-}src}$ activity by phosphorylation-dephosphorylation appears to be cell cycle-dependent (Shalloway *et al.*, 1992; Taylor and Shalloway, 1993). Collectively, these observations support the notion that there is tight regulatory control of activity of *src* gene products *via* direct interaction between Tyr kinases and Tyr phosphatases (Cooper and Howell, 1993).

pp125^{FAK}

The first indication that an "adhesion-related" kinase activity exists in cells came from several reports showing that clustering of integrins enhances Tyr phosphorylation of a 115-130 kDa complex of proteins (Guan *et al.*, 1991; Kornberg *et al.*, 1991). Next, it was shown that cell attachment to ECM-coated substrata triggers the specific phosphorylation of a protein with MW of ~120 kDa, designated as focal adhesion-associated protein Tyr kinase, $pp125^{FAK}$, (Guan and Shalloway, 1992; Hanks *et al.*, 1992; Kornberg *et al.*, 1992). It should be stressed here that $pp125^{FAK}$ phosphorylation is related to the receptor-mediated cell adhesion to ECM as the cell attachment to polylysine-coated substrata has no effect on phosphorylation level of the kinase (Kornberg *et al.*, 1992). $pp125^{FAK}$ does not have SH_2 and SH_3 *src* homology domains characteristic of all of the other members of *src* family of Tyr kinases, and it is neither transmembrane nor membrane-associated protein (Schaller *et al.*, 1992). $pp125^{FAK}$ prominently localizes to focal contacts (Guan *et al.*, 1991; Hanks *et al.*, 1992; Schaller *et al.*, 1992) and is likely to play an important role in their formation and/or maintenance because inhibition of $pp125^{FAK}$ Tyr phosphorylation inhibits formation of focal contacts and stress fibres (Burridge *et al.*, 1992). As $pp125^{FAK}$ is a substrate for $pp60^{v\text{-}src}$ in transformed cells (Kanner *et al.*, 1990; Guan and Shalloway, 1992; Schaller *et al.*, 1992), $pp60^{c\text{-}src}$ might phosphorylate the $pp125^{FAK}$ in normal cells. Hence, it is plausible that this adhesion-related kinase can be regulated both by cell adhesion *via* integrins and *via* oncogenes encoding Tyr kinases (Zachary and Rozengurt, 1992; Schaller and Parsons, 1993).

Transmembrane signalling via adhesions

The association of src Tyr kinases with the plasma membrane positions them well to interact with the cytoplasmic domains of membrane receptors such as integrins that are known to transduce biochemical signals across the cell membrane (Ingber, 1991; Kornberg and Juliano, 1992; Juliano and Haskill, 1993). Indeed, oncogenic viruses that encode Tyr kinases have dramatic effects on cell shape and cell adhesion (Rohrschneider *et al.*, 1982; Burridge *et al.*, 1988; Kellie, 1988; Chen, 1990; Kellie *et al.*, 1991).

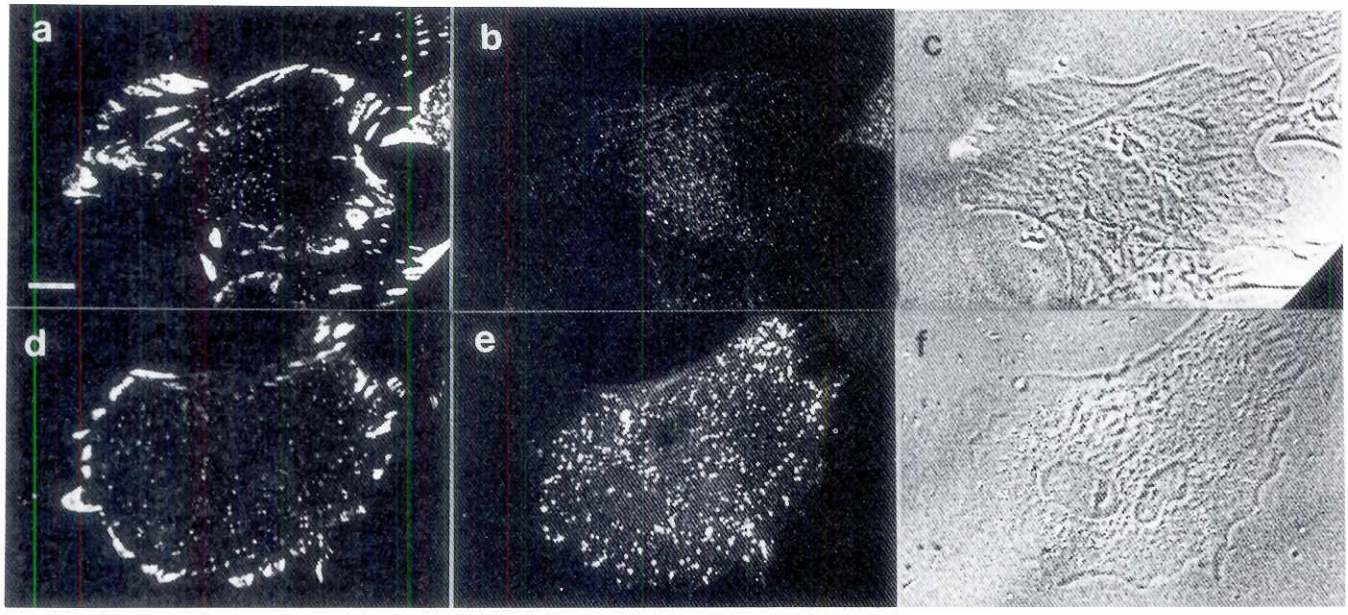

Fig. 2. Confocal immunofluorescence localization of pp125^{FAK} (a,d) and pp60$^{c\text{-}src}$ (b,e) in the same spread, undifferentiated NR (a,b,c) and RPE (d,e,f) cells. (c,f) are corresponding phase contrast photographs. *Cells were fixed with 3.7% paraformaldehyde, permeabilized with 0.5% Triton X-100 and double labelled with anti-pp60$^{c\text{-}src}$ polyclonal antibody followed by Cy3- conjugated donkey anti-rabbit IgGs, and then with anti-pp125^{FAK} monoclonal antibody followed by FITC-conjugated donkey anti-mouse IgGs. The cells were examined using a Bio-Rad MRC-600 confocal laser scanning microscope equipped with Krypton/Argon laser. Focal contacts of well spread NR and RPE cells are highly enriched in pp125^{FAK}* (**a,d** *respectively), while pp60$^{c\text{-}src}$ localizes to submembranous cortex and cytoplasmic vesicles* (**b,e**). *Bar, 10 μm.*

Integrins have relatively short cytoplasmic domains and exhibit no enzymatic activity. Therefore, the mechanisms of signalling *via* integrins must be different from those of growth factor receptors with enzymatically active cytoplasmic domains. Ligand-induced integrin clustering could physically bring together proteins present in focal contact and allow them to interact. Besides containing structural proteins such as actin, α-actinin, talin, and vinculin (a major target of Tyr kinases [Sefton and Hunter, 1981]), focal contacts also contain proteins which may have regulatory functions such as paxillin, which is a major substrate for Tyr kinases and interacts with vinculin *in vitro* (Turner *et al.*, 1990; Burridge *et al.*, 1992), tensin, which has the SH_2 domain and is Tyr-phosphorylated in an adhesion-dependent manner (Davis *et al.*, 1991; Bockholt and Burridge, 1993), and most likely others (Maher *et al.*, 1985; Beckerle *et al.*, 1987). Furthermore, besides pp125^{FAK}, several other kinases, such as an isoform of protein kinase C (Jaken *et al.*, 1989; Hyatt *et al.*, 1990) localize to focal contacts. Because many of the regulatory proteins and even transcription regulators localize to «adhesive» areas of a cell (focal contacts being the best studied example), these structures are involved not only in cell adhesion but also in signal transduction (Ben-Ze'ev, 1991). This would encompass both outside to in signalling initiated by ECM ligand binding, as well as inside to out signalling (Ginsberg *et al.*, 1992; Hynes, 1992; Humphries *et al.*, 1993).

Distribution of non-receptor tyrosine kinases

At the cellular level, the viral and cellular src proteins are associated with plasma membranes, endocytotic vesicles, secretory granules (David-Pfeuty and Nouvian-Dooghe, 1990; Kaplan *et al.*, 1990, 1992) and with motile structures (e.g. filopodia) of neural growth cones in developing neurons (Maness *et al.*, 1988; Sobue, 1990). pp60$^{v\text{-}src}$ is particularly abundant in the detergent-insoluble cytoskeletal matrix of adhesion plaques in transformed cells (Rohrschneider *et al.*, 1982; Kellie *et al.*, 1991). Substantial amounts of both pp60$^{v\text{-}src}$ and pp60$^{c\text{-}src}$ can also be found in association with the nuclear membranes (David-Pfeuty and Nouvian-Dooghe, 1990; Kaplan *et al.*, 1990). pp60$^{c\text{-}src}$, unlike pp60$^{v\text{-}src}$, does not appear in focal contacts and "rosettes", although it does associate with plasma membranes and cell-cell contacts of several cell types in culture (Tsukita *et al.*, 1991). A recent report describes the presence of an intranuclear c-*src* product in keratinocytes (Zhao *et al.*, 1992). The diverse localizations in which src proteins are found imply that these kinases are involved in regulation of many cellular processes.

At the tissue level, the c-*src* gene product is widespread but of interest here is its particular abundance in nervous tissues (Kellie *et al.*, 1991; Brickell, 1992; Maness and Cox, 1992). The highest levels of pp60$^{c\text{-}src}$ have been found in developing neurons of the embryonic brain and retina (Sobue, 1990). pp60$^{c\text{-}src}$ is present in chick pigment epithelium (RPE) both *in vitro* and *in vivo* (Koh, 1992).

pp60$^{c\text{-}src}$ has been localized in developing neurons of chick retina at the onset of differentiation (Sorge *et al.*, 1984; Biscardi *et al.*, 1991). It starts to be expressed at the time when the first neuronal cells (precursors of ganglion and amacrine cells) become postmitotic and migrate toward to the inner surface of the retina. As development proceeds, high levels of pp60$^{c\text{-}src}$ appear both in the

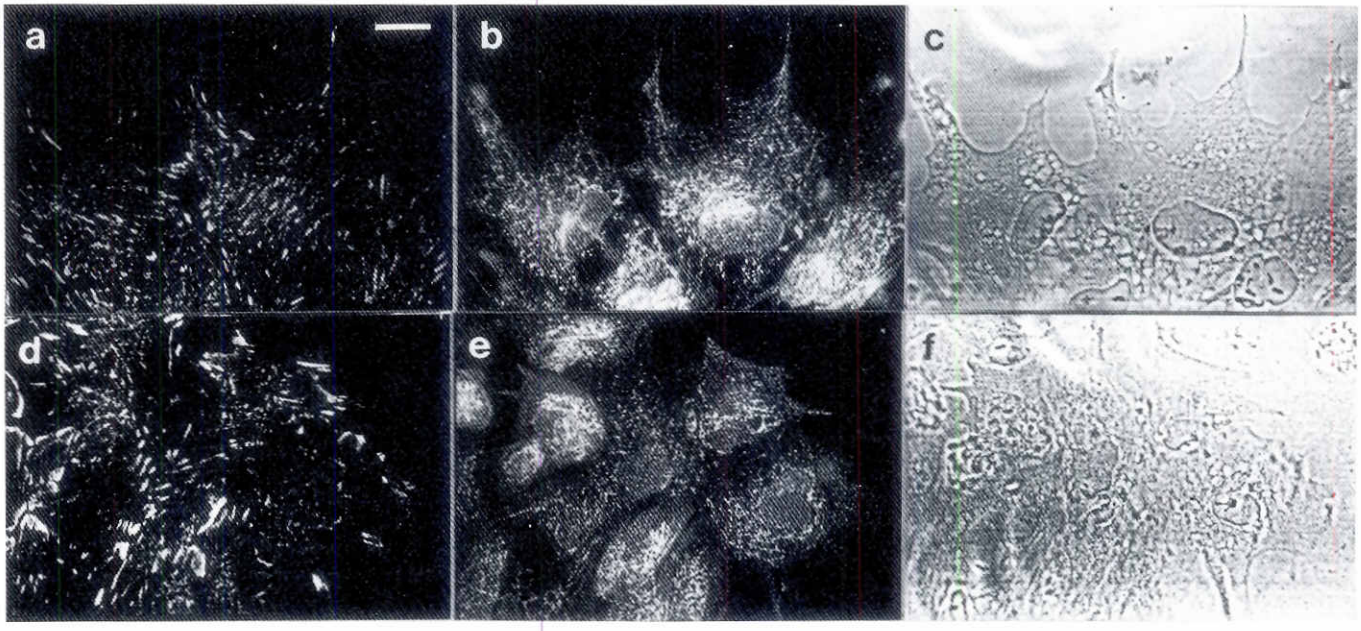

Fig. 3. Confocal immunofluorescence localization of pp125^{FAK} (a,d) and pp60$^{c\text{-}src}$ (b,e) in NR (a,b,c) and RPE (d,e,f) cells at intermediate stages of differentiation *in vitro*. (c,f) are corresponding phase contrast photographs. *Even though the photographs show the most adherent cells from the edges of the colonies, it is evident that in both types of cells the number and size of pp125^{FAK}-positive focal contacts (a,d) have decreased in comparison with the flat spread cells shown in Fig. 2, while pp60$^{c\text{-}src}$ has clearly begun to redistribute to the perinuclear region and the nucleus* **(b,e)**. *Bar, 25 μm.*

ganglion fibre layer and in the inner plexiform layer where the processes of these nerve cells are located. The kinase also seems to be present in the ganglion cell bodies in the ganglion cell layer and in the cell bodies of the amacrine cells in the inner nuclear layer. Later pp60$^{c\text{-}src}$ becomes apparent in the processes of newly developing rods and cones and along the outer limiting membrane, where cell junctions between photoreceptors and the Müller glia are located. The outer portion of the inner nuclear layer is occupied by nuclei of bipolar and horizontal cells. These cells differentiate at approximately the same time as the photoreceptors, however, pp60$^{c\text{-}src}$ could not be detected there at any stage of development or in adult. In the fully differentiated neurons of the adult retina pp60$^{c\text{-}src}$ is present albeit at lower levels (Sorge *et al.*, 1984). Biscardi *et al.* (1991) used antibodies against phospho-tyrosine (P-Tyr) to detect proteins phosphorylated at Tyr residue in the developing eye and optic nerve of stage 34 chick embryos, i.e., when the migration of ganglion cell growth cones reaches maximum but rods and cones have not yet developed. Immunostaining was prominent in layers of retina that have nerve cell processes, i.e., mostly in ganglion fibre layer and inner plexiform layer. This corresponds well to the pattern of pp60$^{c\text{-}src}$ expression described above, although cell bodies did not appear to be stained. The levels of P-Tyr-containing proteins decline with tissue maturation. Recently, several P-Tyr-containing proteins have been detected in the retina of newly hatched chicks (Biscardi *et al.*, 1993). In these studies, Müller glial cells showed high levels of P-Tyr immunoreactivity at sites adjoining photoreceptor inner segments, cell bodies, synaptic terminals and at sites of apposition between plasma membranes of adjacent Müller glial processes.

It appears that the pp60$^{c\text{-}src}$ protein may be more important for neuronal differentiation than proliferation since its appearance is correlated with the onset of histodifferentiation in the retina and since its overexpression does not stimulate retinal cell proliferation (Sorge *et al.*, 1984; Iba *et al.*, 1985; Vardimon *et al.*, 1986, 1991). The fact that c-*src* does not stimulate proliferation is not due to the absence of proliferation-related targets in the neural retina (NR) as the same cells can be effectively stimulated to proliferate by v-*src* (Vardimon *et al.*, 1991; Gillet *et al.*, 1993). This supports the notion that the targets of pp60$^{c\text{-}src}$ are fewer than those of the more promiscuous pp60$^{v\text{-}src}$ (Warren *et al.*, 1988). Alternatively, given the recent data demonstrating that neither c-*src* knockout (Soriano *et al.*, 1991) nor c-*src* overexpression (Vardimon *et al.*, 1991) have any detectable effect on development and function of neural tissue, a possibility emerges that the c-src kinases are either neural "junk proteins" (Erickson, 1993), i.e., proteins superfluously expressed in neural tissue or that under knockout conditions, other mechanisms and/or proteins take over src functions. Clearly, identification of pp60$^{c\text{-}src}$ substrates is key to understanding its role in neuronal cells in general and in differentiation of retina in particular.

pp125^{FAK} is an ubiquitous kinase since it is present in every tissue examined so far. The amount of kinase expressed is regulated during chick development: its abundance is high in the first half of embryogenesis and declines during its second half to much lower levels (Turner *et al.*, 1993). Interestingly, the level of pp125^{FAK} phosphorylation is also developmentally regulated and increases initially to reach the highest levels during the middle third of embryogenesis, and then declines towards hatching (Turner *et al.*, 1993).

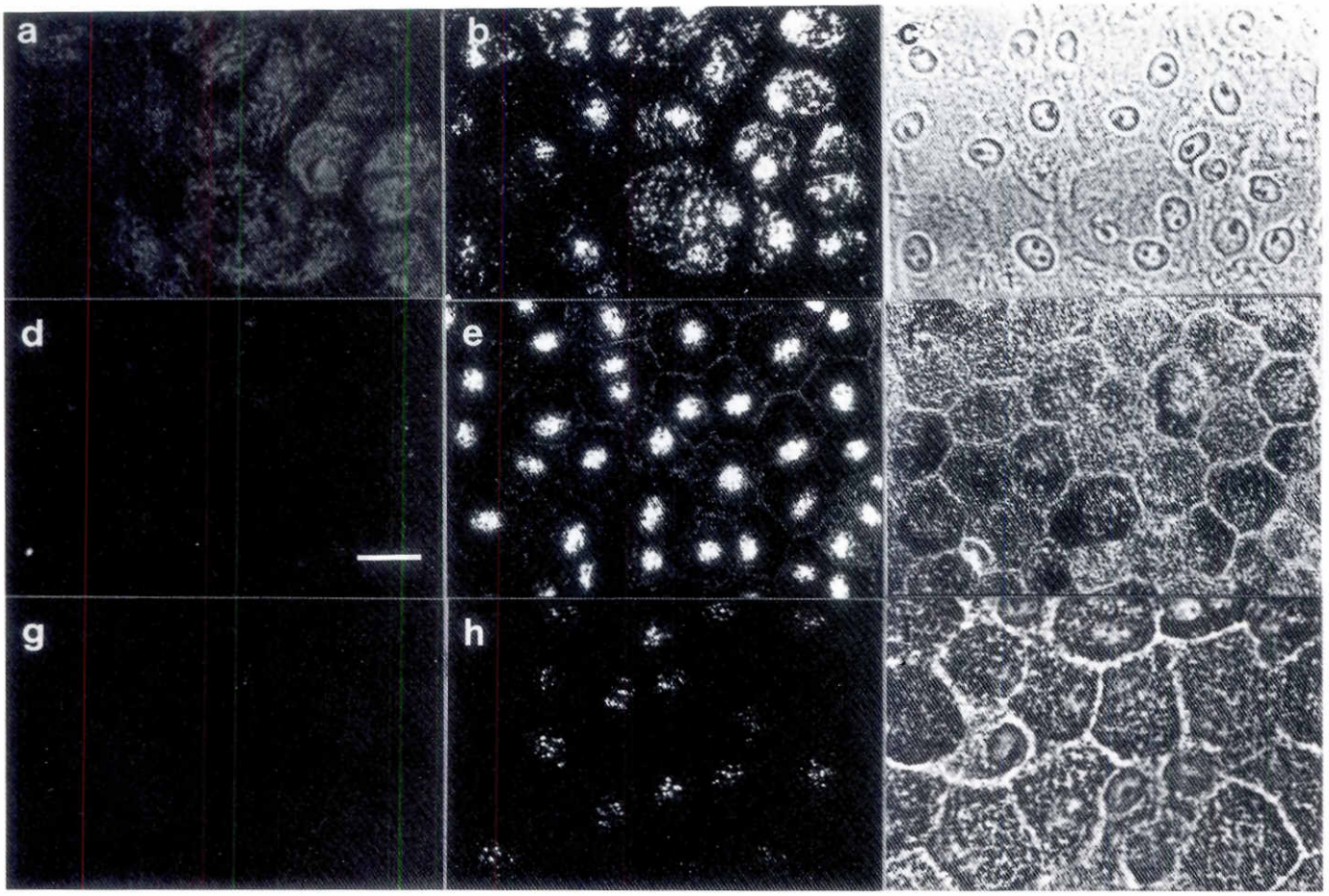

Fig. 4. Confocal immunofluorescence localization of pp125^{FAK} (a,d,g) and pp60$^{c\text{-}src}$ (b,e,h) in packed NR (a,b,c) and RPE (d,e,f) cells which have attained differentiated phenotype *in vitro*. (g,h,i) *show RPE cells derived from the NR by transdifferentiation in vitro.* **(c,f,i)** *are corresponding phase contrast photographs. In both NR and RPE cells that are derived by redifferentiation of initially dedifferentiated respective cell types as well as in transdifferentiating cells pp125^{FAK}-positive focal contacts are absent and the nuclear localization of pp60$^{c\text{-}src}$ is predominant in cells of the NR* **(b)** *and exclusive in cells of the RPE* **(e,h).** *In RPE cells at this stage of differentiation in vitro pp60$^{c\text{-}src}$ is also abundant in junctional complexes* **(e)**. *Bar, 10 μm.*

Regulation of phenotypic expression of retinal cells *in vitro*

The RPE *in vivo* is a monolayer of tightly adhered cells that rests on a basement membrane (BM). Embryonic RPE cells differentiate *in vitro*, i.e., pack into an epithelial sheet (Crawford, 1979; Owaribe *et al.*, 1981), and acquire specific RPE markers (Chu and Grunwald, 1990) and pigmentation (Crawford, 1979; Opas *et al.*, 1985; Opas and Dziak, 1988). During their differentiation *in vitro* the RPE cells display a differentiation-dependent organization of the cytoskeleton, adhesiveness and ECM production (Crawford, 1979, 1980; Turksen *et al.*, 1983, 1984, 1987; Crawford and Vielkind, 1985; Opas and Kalnins, 1985; Opas *et al.*, 1985; Opas, 1989; Owaribe, 1990; Rizzolo, 1991). In the RPE culture systems it is possible, by manipulating environmental conditions such as properties of the substratum and soluble factors, to control the proliferative behaviour and the determination of the choice of fate by the presumptive RPE (Yasuda, 1979; Itoh and Eguchi, 1986a,b; Reh *et al.*, 1987; Kosaka *et al.*, 1992; Hyuga *et al.*, 1993; Opas and Dziak, 1994).

The NR, when grown *in vitro*, forms epithelial sheets comprising flat cells. Growing on top of the flat cells are neuroblastic cells which, under standard culture conditions, are lost within a few weeks *in vitro* (Combes *et al.*, 1977; Li and Sheffield, 1986a,b). These cells which flattened out proliferate and give rise to the epithelial sheets that are glial and are derived from the same progenitor cells as the Müller cells (Li and Sheffield, 1984; Moyer *et al.*, 1990).

Transdifferentiation

Transdifferentiation (also known as "cell type conversion") is the process by which the differentiated cells change their identity from one distinct cell type to another (Okada, 1986; Eguchi and Kodama, 1993). Transdifferentiation occurs in several systems (Parker *et al.*, 1980; Moscona and Linser, 1983; Lopashov and Zviadadze, 1984; Eguchi, 1986, 1988; Okada, 1986; Schmid and Alder, 1986; McDevitt, 1989; Beresford, 1990; Watt, 1991; Schmid, 1992) and in neural tissues is thought to be closely related to the capacity for regeneration (Hitchcock and Raymond, 1992). The embryonic

retina has been extensively studied because of its remarkable phenotypic plasticity (Okada *et al.*, 1979; Okada, 1980; Moscona, 1986; Eguchi and Kodama, 1993; Okada and Yasuda, 1993). In a pioneering work Eguchi and Okada (1973) provided unequivocal evidence that a clonal population of RPE cells can transdifferentiate into lens. Subsequently, Eguchi and his collaborators showed that the potential to transdifferentiate into lens also resides in iris epithelium (Eguchi *et al.*, 1974; Yasuda *et al.*, 1978; Eguchi, 1979). The RPE is at least bipotential as it can transdifferentiate into either lens epithelium (Eguchi, 1986; Itoh and Eguchi, 1986b; Kosaka *et al.*, 1992; Agata *et al.*, 1993; Okada and Yasuda, 1993) or neuroepithelium (Coulombre and Coulombre, 1965; Okada, 1980; Tsunematsu and Coulombre, 1981). In an early report Barr-Nea and Barishak (1972) also described formation of a stratified squamous epithelium from RPE. The flat glial cells of cultured NR may convert into melanin-producing RPE cells (Okada *et al.*, 1979; Okada, 1980; Pritchard, 1981). More often, however, and especially in the presence of insulin, the NR transdifferentiates directly into lens epithelium (Okada *et al.*, 1979; de Pomerai and Clayton, 1980; Okada, 1980; Moscona and Degenstein, 1981; de Pomerai *et al.*, 1982; Moscona, 1986; Okada and Yasuda, 1993). Transdifferentiation of the RPE into the NR has been induced by delivering basic fibroblast growth factor (bFGF) into the embryonic eye after microsurgical removal of the NR (Park and Hollenberg, 1989, 1991). Subsequently, it was shown that bFGF also promotes the *in vitro* transdifferentiation of RPE into NR (Pittack *et al.*, 1991; Guillemot and Cepko, 1992; Opas and Dziak, 1994) and enhances the transdifferentiation of RPE into lens (Hyuga *et al.*, 1993).

Transdifferentiation may occur either by a direct change of one cell type into another without DNA synthesis and cell proliferation (one-step or direct transdifferentiation) or indirectly, after going through intermediate stages formed as cells undergo DNA synthesis and proliferation (multi-step transdifferentiation) (Okada, 1986; Beresford, 1990; Schmid, 1992). Transformed neuroectodermal cells transdifferentiate *in vitro* (Ciccarone *et al.*, 1989) and inhibition of their proliferation (by suppression of *myc* proto-oncogene) restricts the ability of these cells to transdifferentiate (Whitesell *et al.*, 1991a,b). Transdifferentiation of embryonic RPE into NR requires an extensive cell proliferation and thus appears to be a multi-step transdifferentiation (Coulombre and Coulombre, 1965; Park and Hollenberg, 1989; Pittack *et al.*, 1991; Opas and Dziak, 1994).

It has been postulated that transdifferentiation of either the NR or RPE into the lens epithelium occurs by direct transdifferentiation (Moscona *et al.*, 1983). This possibility also derived support from the finding that transdifferentiation of NR into lens occurs not as a result of activation of inactive genes, but by enhancing the expression of genes that are already expressed albeit at low levels (de Pomerai and Clayton, 1978; Errington *et al.*, 1985; Kondoh and Okada, 1986; Kondoh *et al.*, 1987). In an elegant series of studies Eguchi's group (Mochii *et al.*, 1988a,b; Agata *et al.*, 1993), however, has shown that transdifferentiation of RPE into lens proceeds *via* an intermediate, dedifferentiated cell type in which the c-*myc* gene is activated but neither the RPE- nor lens-specific genes are. Expression of residual amounts of some crystallins, proteins specific to the differentiated lens cells, has been reported not only in the NR (Head *et al.*, 1991) but also in the RPE (Reddy *et al.*, 1991). The association between the expression of crystallins in non-lenticular cells and the potential to transdifferentiate into lens apparently exists, but the basis for this association is still far from clear. The expression of δ-crystallin in the NR and RPE cells differs from its expression in the lens not only in terms of lower protein levels in the former, but also in terms of their regulation: δ-crystallin accumulation is much greater in transdifferentiating cultures of early embryonic NR whereas α- and ß-crystallins become more prominent in cultures of late embryonic NR (de Pomerai and Clayton, 1978).

Adhesion, cell shape, tyrosine phosphorylation and phenotypic expression in vitro

Adhesiveness of an epithelial cell is regulated by the surface properties of its neighbours and by the nature of the ECM (Adams and Watt, 1993; Hay, 1993) which, to a large extent, determines cell shape (Watt, 1986; Stoker *et al.*, 1990). These regulatory effects of adhesiveness have been demonstrated during transdifferentiation of a variety of cell types (Pritchard *et al.*, 1978; Moscona *et al.*, 1983; Ophir *et al.*, 1985; Moscona, 1986; Boukamp and Fusenig, 1993; Schmid *et al.*, 1993), including RPE (Yasuda, 1979; Eguchi *et al.*, 1982; Reh *et al.*, 1987; Opas and Dziak, 1994). As previously mentioned, the soluble factor, FGF, is instrumental in transdifferentiation of chick RPE into the NR both *in ovo* and *in vitro*. FGFs, their mRNAs and their receptors are abundant in NR and RPE of diverse species (Jeanny *et al.*, 1987; Baudouin *et al.*, 1990; Cirillo *et al.*, 1990; Fayein *et al.*, 1990; Heuer *et al.*, 1990; Jacquemin *et al.*, 1990; Mascarelli *et al.*, 1991; Wanaka *et al.*, 1991; Gao and Hollyfield, 1992; Ishigooka *et al.*, 1992). FGFs are bound to heparan sulfate proteoglycans in a variety of BMs (Moscatelli *et al.*, 1991), including the BM of the RPE (Jeanny *et al.*, 1987). Taken together, these data suggest that both soluble factors (e.g., FGFs) and adhesion *via* either attachment factors (e.g., ECM) or cell-cell interactions, play a pivotal role in determining cell fate choice during retinal differentiation.

In both RPE and NR, the cell shape is controlled by strong adhesions that are realized by two subclasses of adherens junctions: focal contacts and zonulae adherens. The fact that protein phosphorylation on Tyr affect the stability of focal contacts (Burridge *et al.*, 1992) and the existence of the focal contact specific kinase (Schaller and Parsons, 1993) allow for some inferences as to the importance of kinases in the cell-substratum adhesion. As far as the cell-cell adhesion is concerned, while the dependence of zonulae adherens stability on the Tyr phosphorylation levels is quite well established (see Introduction), the role of src kinases there is far from clear. Our immunolocalization studies show that $pp60^{c\text{-}src}$ distributes similarly in the NR and the RPE cells during their differentiation *in vitro*. While the protein localizes to submembranous cortex and vesicles in the flat undifferentiated cells (Fig. 2) it progressively becomes more intranuclear as the cells start to differentiate and pack more closely together (Fig. 3). In fully packed differentiated cells the $pp60^{c\text{-}src}$ is predominately intranuclear (Fig. 4). The intranuclear localization of $pp60^{c\text{-}src}$ is rather unusual. The redistribution of $pp60^{c\text{-}src}$ in our retinal cell cultures resembles, however, the redistribution of $pp60^{c\text{-}src}$ from the cell surface to the nucleus during differentiation of keratinocytes (Zhao *et al.*, 1992). In summary, in the retinal cultures, the differentiation-associated redistribution of $pp60^{c\text{-}src}$ is accompanied by downregulation of $pp125^{FAK}$ and a switch from predominantly cell-substratum adhesion associated with focal contacts to cell-cell adhesion mediated by zonulae adherens.

Interestingly, we detect the intranuclear presence of $pp60^{c\text{-}src}$ also in the NR cells that transdifferentiated into the RPE *in vitro* (Fig.

4). Even more curiously, the cell-cell junctional complexes are enriched in pp60$^{c\text{-}src}$ in the RPE cells at intermediate-to-late stages of differentiation *in vitro*. This enrichment occurs irrespective of whether the RPE cells are derived by redifferentiation of dedifferentiated RPE or by transdifferentiation of NR. In fact, an increase in Tyr-phosphorylation of several major proteins during differentiation has been detected in junction-rich areas of the retina (Shores and Maness, 1989). pp60$^{c\text{-}src}$ is also elevated in transdifferentiating NR cells that have committed to lens fate (Ellis *et al.*, 1987). The data of Ellis *et al.* (1987) parallel our findings in that the highest levels of pp60$^{c\text{-}src}$ are found in those cells that are at intermediate-to-late stages of transdifferentiation, i.e., are not the NR already, but are not lens epithelium yet. Then what may be the role of the transient increase of pp60$^{c\text{-}src}$ abundance in junctions of differentiating and/or transdifferentiating cells? The overexpression of pp60$^{c\text{-}src}$ does not appear to phosphorylate and degrade the adherens junctions (Warren *et al.*, 1988; Behrens *et al.*, 1993). It has been reported, however, that epithelial cells expressing elevated levels of pp60$^{c\text{-}src}$ are less rigid than their normal counterparts (Warren *et al.*, 1988). The interesting conjecture can therefore be made that modulation of cell rigidity by pp60$^{c\text{-}src}$ is at play during transdifferentiation of retinal cells. In fact, during spontaneous transdifferentiation of RPE into NR, the FGF-responsive cells are always "squeezed out" by their neighbors and protrude above the cell sheet (Zhou and Opas, submitted). Collectively, it is recently becoming more obvious that mechanical properties of cells and of their extracellular environment [i.e., cytomechanics (Opas, 1987]) play an important role in regulating the phenotypic expression in transdifferentiating cell systems (Opas, 1989, 1994; Schmid, 1992; Schmid *et al.*, 1993; Opas and Dziak, 1994).

To differentiate, epithelial cells withdraw from cell cycle and reorganize their cytoarchitecture from a "spread" to a "round" phenotype. These structural changes activate the expression of tissue-specific genes, by a process in which the cell structure is both the signal and its medium. The transmembrane linkage complexes and growth factor receptors cooperate in transducing signals outside-in and inside-out of the cell via activation of protein kinases. Finally, protein kinases appear to exert extensive control over the cell shape, cell contacts and the cytoskeleton. Phosphorylation, such as that initiated by the binding of FGF to its receptor or by the clustering of integrins, plays an important role in signal transduction starting a chain of events leading to DNA synthesis. The involvement of similar mechanisms in transdifferentiation seems very likely since transdifferentiation can be regarded as a "new", redirected differentiation (Nathanson, 1986). Thus, as in cell differentiation and morphogenesis (Zelenka, 1990; Birchmeier e*t al.*, 1993), proto-oncogenes and Tyr kinases may also play a key role in cell transdifferentiation.

Summary

Regulation of phenotypic expression in epithelia in general, and of two epithelia of the retina, the neural retina and retinal pigment epithelium in particular, is dependent on interactions with extracellular environment. Extracellular environment may comprise acellular substrata as well as other cells. Non-receptor protein tyrosine kinases are involved in transmembrane transmission of signals from extracellular milieu, via the cytoskeleton to the nucleus. We describe distribution of these kinases in cells of retinal origin and show that two of them, pp125^{FAK} and pp60$^{c\text{-}src}$ redistribute intracellularly in a differentiation-dependent manner. Next we discuss roles that adhesion-related non-receptor protein tyrosine kinases might play in phenotypic expression by the retinal epithelia.

KEY WORDS: *retinal pigment epithelium, retina, tyrosine kinases, adhesion, differentiation, transdifferentiation*

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