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Feature Articles

The S-100-related Calcium-binding Protein, p9Ka, and Metastasis in Rodent and Human Mammary Cells

R. Barraclough and P.S. Rudland

INTRODUCTION

METASTASIS, the process whereby cancer cells spread from their site of origin to distant sites within the body, is responsible for the majority of deaths from solid cancers such as those of breast, lung, colon and prostate. In order to understand more fully the metastatic process, changes in gene expression in metastatic cells have been sought. Many such changes in metastatic cells result in an increase in expression of proteins which are associated with processes such as proteolysis and cell motility, and a reduction in proteins associated with processes such as cell adhesion [1–3]. However, it is an understanding of the co-ordination and regulation of such cellular processes that is likely to be important in understanding the primary lesions in a metastatic cell. Many intracellular processes are regulated in a highly specific manner by calcium ions acting through calcium-binding proteins. This article concerns a calcium-binding protein which can induce the metastatic phenotype in rat mammary tumour cells.

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UPREGULATION OF p9Ka AND ITS mRNA IN METASTATIC RODENT MAMMARY CELLS

p9Ka was first discovered as a polypeptide which is induced when certain cultured rat mammary epithelial cells change to an

elongated myoepithelial-like cell [4]. However, elevation of p9Ka or its mRNA accompanies other induced changes in tissue culture cells, namely, nerve growth factor-induced differentiation of rat PC12 pheochromocytoma cells [5], serum-induced increase in growth rate of culture murine fibroblasts [6] and oncogene- or carcinogen-induced transformation of murine fibroblasts [7] or rat kidney cells [8]. These observations have suggested an association of p9Ka and the growth and differentiation of such cells. However, elevated levels of p9Ka and its mRNA have also been found in a metastatic rat mammary epithelial cell line [9], relative to its non-metastatic epithelial counterparts both from benign tumours [10], and from normal rat mammary glands [11]. Furthermore, in a series of closely-related murine mammary adenocarcinoma cell lines, which differ in their potential to spread to the lungs after intramuscular injection into recipient mice, there was a quantitative correlation between their metastatic potential and the level of expression of the mRNA for p9Ka [12]. These correlations are not confined to mammary cells, since the level of the mRNA for p9Ka also shows a strong correlation with the ability of murine metastatic B16 melanoma cells to colonise the lungs of recipient mice [13]. These correlations between the level of p9Ka or its mRNA and the ability of rodent cells to metastasise have been investigated further using a rat mammary model system.

AN *IN VIVO* ASSAY FOR METASTASIS-INDUCING GENES AND THEIR PRODUCTS

The metastatic potential of rat mammary cultured cell lines is conveniently tested by injecting cells into the mammary fat pads of syngeneic rats, and observing the appearance of tumours both at the site of injection and in remote locations such as lungs and lymph nodes. When epithelial cell lines derived from metastatic rat mammary tumours are tested, tumours that arise at the site of injection spread to the lungs and lymph nodes of the recipient rats [14, 15], whereas those from benign tumours do not [16]. However, these same benign mammary epithelial cell lines can acquire the ability to disseminate if they are first transfected with DNA isolated from rat or human metastatic cells. To select for those cells which have taken up the DNA, the cells are co-transfected with a plasmid, pSV2*neo* [17], and the transfected cells selected by their ability to grow in the presence of the antibiotic geneticin, prior to being injected into the mammary fat pads of recipient rats [16, 18, 19]. Transfection of the benign cells with the selective plasmid alone, or with plasmid plus DNA from normal tissue or benign tumours all fail to induce the metastatic phenotype. This syngeneic rat model (Figure 1) provides a system in which the protein products of cloned genes, such as p9Ka, can be tested for their ability to induce the metastatic phenotype in rodent cells.

p9Ka CAN INDUCE THE METASTATIC PHENOTYPE IN NON-METASTATIC RAT MAMMARY TUMOUR CELLS

Multiple additional (10–100) expressed copies of the cloned rat p9Ka gene [20] have been transfected into the benign tumour cell line, Rama 37 [10]. The cloned DNA fragment used for the transfections contained 10.3 kbp of cloned normal rat DNA, containing the p9Ka gene (2.3 kbp), and approximately 8 kbp of flanking DNA sequences. Prior to being transfected, the p9Ka gene was covalently linked to the selective plasmid, pSV2*neo* [17]. The resulting stably-transfected, geneticin-resistant cells were injected into the mammary fat pads of syngeneic rats which were then examined for the appearance of metastases [21].

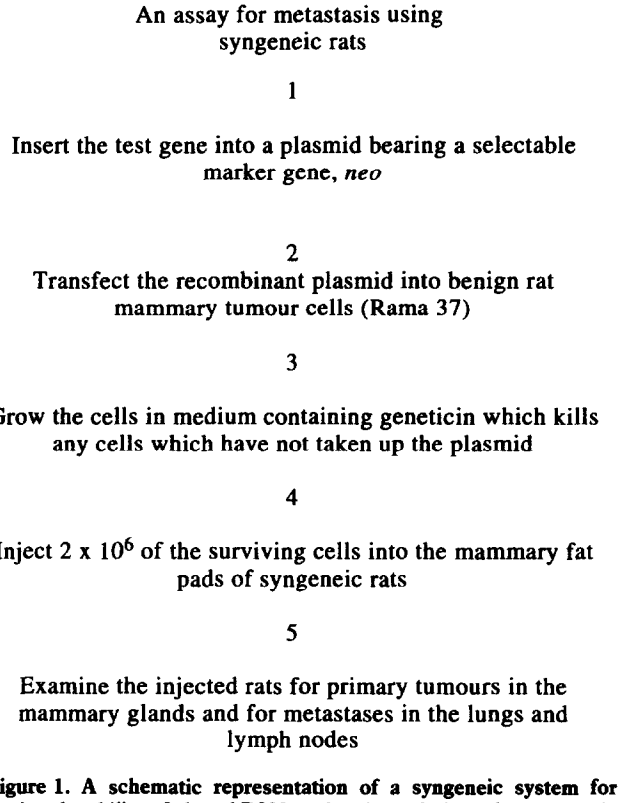

Figure 1. A schematic representation of a syngeneic system for testing the ability of cloned DNA molecules to induce the metastatic phenotype.

Untransfected recipient cells produce tumours in 50% of injected rats, but no metastases (Table 1). When cells transfected with plasmid containing the entire p9Ka gene [20] were injected into the recipient rats, there was an increase in tumour incidence to nearly 100%, and a 2–3-fold reduction in the latent period of tumour development [21], extending the initial correlations between p9Ka mRNA expression and growth rate of cultured cells [6, 8]. Most interestingly, in over 50% of the animals, the tumours disseminated to the lungs and lymph nodes [21]. This acquisition of the metastatic phenotype is unlikely to be due to the additional copies of the p9Ka gene *per se*, since integration into the DNA of the benign tumour cells (Rama 37) of similar numbers of copies of the plasmid, pSV2*neo* carrying the selectable marker, or the pSV2*neo* plasmid containing an activated c-Ha-*ras*-1 gene [22], failed to induce the metastatic phenotype (Table 1) [21]. The primary tumours and metastases arising from the cells transfected with the p9Ka gene (Figure 2c) contained elevated levels of p9Ka, detected immunocytochemically [21], suggesting that it is the overproduction of p9Ka that is responsible for the metastatic potential in these transfected mammary epithelial cells as well as in the p9Ka-expressing metastatic cells from rats [9] and mice [12, 13]. In the mammary cells transfected with the p9Ka gene, the metastatic phenotype was stable in cells isolated and cultured from metastases in the lungs and lymph nodes. Upon injection of these cultured metastatic cells into the mammary fat pads, there was a similar incidence of metastases to that obtained with the primary transfectants.

p9Ka IS A CALCIUM-BINDING PROTEIN

The gene for rat p9Ka [20] contains three exons of 37, 156 and 295 nucleotide pairs and two intervening sequences of 1172 and 675 nucleotide pairs (Figure 3). Exons two and three encode

Table 1. Incidence of tumours and metastases produced by subcutaneous injection of mammary epithelial cells and transfected derivatives [21]

Transfected DNA	Number of tumours/ number of sites inoculated*	Median latent period in days (range)	Number of animals with metastases/ number of animals with tumours
None (untransfected Rama 37 cells)	22/46	33 (24–81)	0/20
pSV2*neo*-p9Ka			
Experiment 1	19/19	12 (6–28)†	9/15‡
Experiment 2	18/18	8 (7–11)†	4/9
Experiment 3	7/10	15 (8–27)†	3/5
Total	44/47	—	16/29‡
pSV2*neo*	6/15	29 (14–43)	0/6
pSV2*neo-ras*	10/25	20 (16–43)	0/10

*Benign tumour-derived epithelial cells, Rama 37, were transfected with plasmid pSV2*neo*, or pSV2*neo* containing the activated c-Ha-*ras*-1 gene or the rat p9Ka gene. The resulting transfected cells were injected, subcutaneously into the mammary fat pads of recipient rats. † Significantly shorter latent period than Rama 37 cells ($P < 0.01$; Mann-Whitney U-test). ‡ Significantly more metastases than Rama 37 cells ($P < 0.05$; Fisher exact test).

the translated region of the p9Ka mRNA, whereas the small 5′ exon gives rise to an untranslated region of the mRNA, and the intervening sequence which separates it from exon two may have regulatory significance, since it contains DNA which can stimulate the expression of a chloramphenicol acetyl transferase reporter gene, and specific sequences of DNA which can bind nuclear proteins, when tested in mobility-shift and DNA-footprinting assays [23].

The nucleotide sequence of the rat p9Ka gene contains a potential coding region of 101 amino acids (Figure 4), including the initiating methionine residue [20]. Regions of this derived amino acid sequence correspond exactly to the amino acid sequence of proteolytic fragments of purified natural p9Ka (Figure 4). p9Ka exhibits 27% similarity of amino acid sequence to the rat vitamin D-dependent, intestinal calcium-binding protein [24], greater than 40% similarity to members of the S-100 family of EF-hand-containing proteins, including the α and β subunits of S-100 [25, 26], and the growth-regulated calcyclin [27], and 32% similarity with p11, the small subunit of annexin II [28]. In common with these proteins, p9Ka contains two potential EF-hand calcium-binding sites. The C-terminal loop (residues 62–73) of p9Ka corresponds to an almost perfect EF-hand sequence [29], whereas, the N-terminal, potential calcium-binding region is a variant EF-hand loop typical of that in the S-100 proteins [30].

The ion-binding properties of p9Ka have been studied using both natural p9Ka purified by high-performance liquid chromatography [31] and recombinant p9Ka (rp9Ka) produced in *E. coli* cells [32, 33] at a level of 40–50 mg/l [34], and purified using a rapid, two-step procedure [34]. rp9Ka binds two calcium ions per molecule with a Kd of 34 ± 0.3 to 38 ± 0.6 over a 10-fold range of rp9Ka concentrations [34], which is similar to the reported value of 76 ± 14 μM for natural p9Ka [31]. The affinity of p9Ka for calcium ions determined *in vitro* is less than that of high-affinity, regulatory calcium ion-binding proteins such as calmodulin (3–20 μM) [34, 35], calcium storage proteins such as parvalbumin (0.1 μM) [36], and the calcium ion-transporting vitamin D-dependent intestinal calcium ion-binding protein (2 μM) [37]. However, p9Ka has an affinity for calcium ions which is in the same range as that of other closely-related, small calcium ion-binding proteins of the S-100 protein family for which calcium binding has been determined [38]. The binding of calcium by p9Ka *in vitro* is strongly antagonised by the presence of physiological concentrations of sodium, potassium or magnesium ions in the medium [34], a property which p9Ka shares with other S-100 proteins [39]. If the binding affinities measured *in vitro* reflect those *in vivo*, it is possible that p9Ka binds calcium in a location in which calcium ions are either elevated above normal levels, or where the potassium ion concentration is low.

p9Ka IS A WIDELY DISTRIBUTED PROTEIN WHICH SHOWS AN UNUSUAL TOPOGRAPHICAL LOCATION IN THE RAT MAMMARY GLAND

Experiments in different laboratories have sought to establish the normal tissue distribution of the mRNA for p9Ka in rats and mice. These experiments, employing cloned cDNA molecules [6, 12, 40], have not shown a consistent pattern of expression in normal tissues. In the mouse mRNA corresponding to p9Ka has been detected in spleen, bone marrow, thymus and lymphocytes [12]. However, others, using a mouse p9Ka cDNA, designated 18A2, detected p9Ka mRNA in uterus, placenta and kidney with very low levels being detected in the thymus and testes [6]. In the rat, the mRNA for p9Ka has been detected in the spleen, and low levels have been found in the mammary gland and uterus, with variable levels being reported in liver [41]. These variable levels of the mRNA for p9Ka reported by different laboratories may be a consequence of its presence in some blood cells which may contaminate isolated normal tissues [12, 41].

In order to study in more detail the immunocytochemical location of p9Ka, an antiserum to rp9Ka was raised in rabbits. This antiserum reacts specifically with p9Ka [41] and does not react even with closely-related calcium-binding proteins, calcyclin [42], S-100 protein subunit or MRP8 or MRP14 [43]. In solid tissues of the rat, p9Ka is not detected immunocytochemically, in mitotically-active cells where these are identifiable, such as in the crypts of the intestine and in the roots of the hair follicles of the skin. However, p9Ka is widely expressed in differentiated epithelial, neuronal and muscle cells. For example, in the stomach, staining is present over the acid-secreting

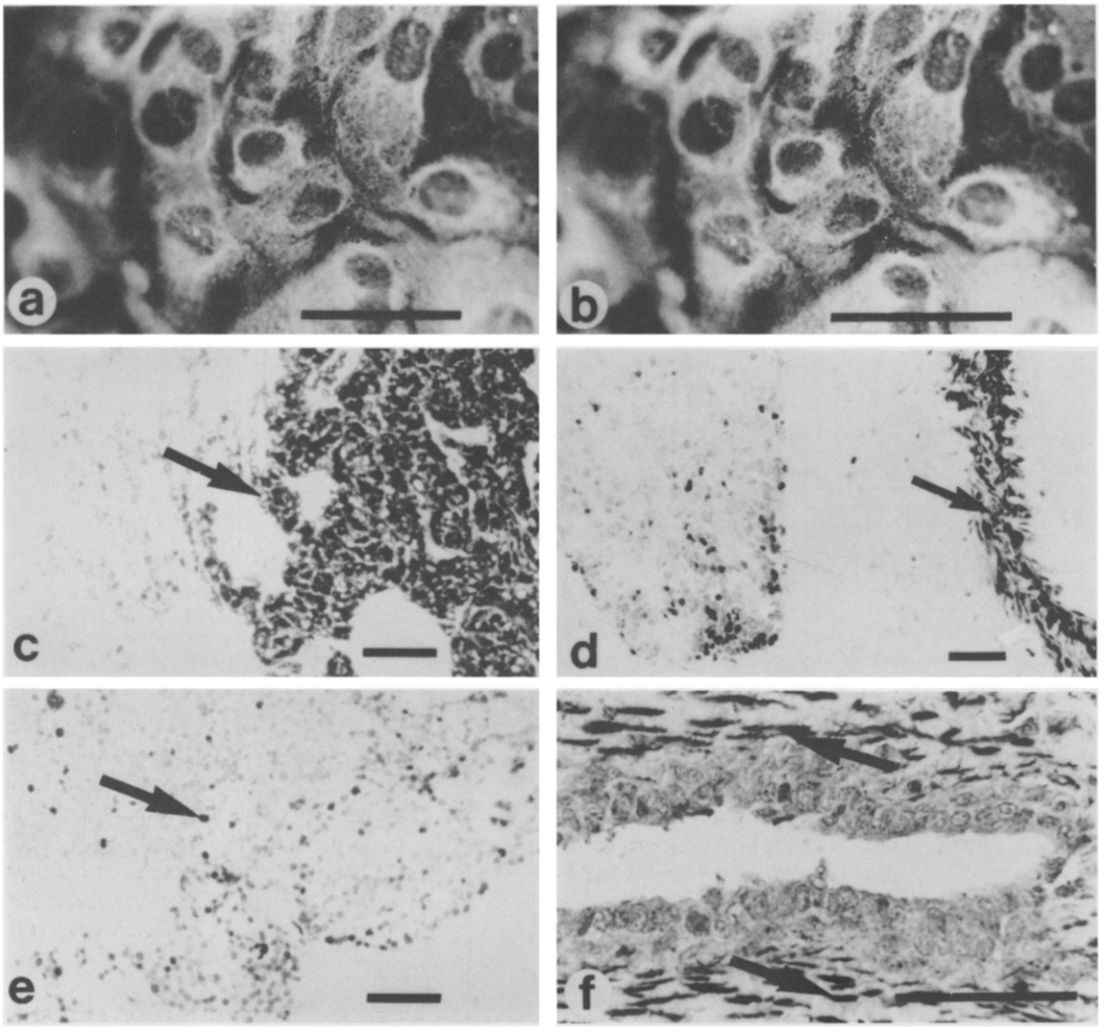

Figure 2. The immuofluorescent and immunocytochemical distribution of p9Ka in rat cells and tissues. (a) Cuboidal epithelial cells transfected with the p9Ka gene stained with FITC-labelled anti-p9Ka serum. (b) The same field as in (a) but stained with TRITC-labelled phalloidin to visualise the actin/myosin cytoskeleton. (c) A lung metastasis arising from p9Ka transfected cells; the tumour on the right (arrow) stains strongly with anti-p9Ka serum, whereas the normal lung tissue stains relatively weakly. (d) The smooth muscle of a blood vessel (arrow) immunocytochemically stains strongly with anti-p9Ka serum. (e) A proportion of small nucleated cells (arrow) in the rat bone marrow stain strongly for p9Ka. (f) In the mammary gland, wavy staining for p9Ka is observed (arrows) which does not correlate with any particular cell type. Magnifications were (a), (b) and (f) × 580; (c) and (e) × 230; (d) × 185. The bars under each figure represent 50 μm.

parietal/oxyntic cells, but there is no staining over the chief/peptic cells. In the intestine, staining is mainly in the columnar absorptive cells and neuronally-derived Auerbach's plexus, and in the lung there is staining of alveolar septa and in the smooth muscle of blood vessels (Figure 2c, d). p9Ka is present in a proportion of small nucleated cells in the marrow (Figure 2e) and spleen, in endothelial cells and also within some of the cells of the terminal end buds of growing ducts in the developing rat mammary gland. It is interesting to note that these cells possess potentially invasive properties as the ducts extend into the fat pads. This staining appears to be intracellular.

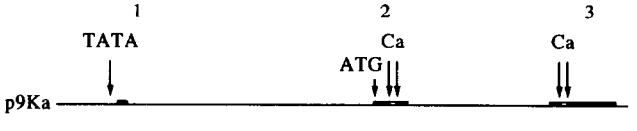

Figure 3. The intron/exon structure of the rat p9Ka gene. The three exons are indicated by the black boxes labelled 1, 2 and 3. The regions of the exons encoding the calcium-binding sites in exons 2 and 3 are indicated between the arrows by the white boxes labelled Ca.

p9Ka is not detectable within the parenchymal cells of the adult mammary gland. However, in the fully-developed mammary gland, there is an intense wavy staining close to, but separate from the parenchyma, and the staining is not associated with identifiable cells (Figure 2f). A similar pattern of staining is observed in some other tissues, notably spleen and brain. In the mammary gland, the staining resembles that of elastic fibres, however, when adjacent histological sections of rat mammary gland are stained immunocytochemically for p9Ka and histologically for elastic fibres, the pattern of staining is similar, but not identical (Gibbs *et al.*, submitted for publication). In part, this lack of identity may be due to the size of elastic fibres relative to the thickness of the histological sections. Thus, currently, the exact structures which stain for p9Ka in the rat mammary gland (and also in spleen and brain tissue) remain to be conclusively identified.

EF - hand													n			n	f			n	O	
Rat p9Ka	1	M	A	R	p	L	E	E	A	L	D	V	I	V	S	T	F	H	K	Y	S	G
Human p9Ka	1	M	A	c	p	L	E	k	A	L	D	V	m	V	S	T	F	H	K	Y	S	G
Calcyclin	1	M	A	c	p	L	d	q	A	i	g	l	l	V	a	i	F	H	K	Y	S	G
Rat S - 100	1	M		s	e	L	E	k	A	m	v	a	l	i	d	v	F	H	q	Y	S	G
Rat intestinal	1												m	k	S	i	F	q	K	Y	a	a
Pig P11	1	M	p	s	q	m	E	h	A	m	e	t	m	m	f	T	F	H	K			
EF - hand			0		O			O	L		K		E	L	K		L	n			E	
Rat p9Ka	22	N	E	G	D	K	F	K	L	N	K	T	E	L	K	E	L	L	T	R	E	L
Human p9Ka	22	k	E	G	D	K	F	K	L	N	K	s	E	L	K	E	L	L	T	R	E	L
Calcyclin	22	r	E	G	D	K	h	t	L	s	K	k	E	L	K	E	L	I	q	k	E	L
Rat S - 100	21	r	E	G	D	K	h	K	L	k	K	s	E	L	K	E	L	I	n	n	E	L
Rat intestinal	11	k	E	G	D	p	n	q	L	s	K	e	E	L	K	l	L	I	q	s	E	f
Pig P11	19	f	a	G	D	K	g	y	L	t	K	e	d	L	r	v	L	m	e	k	E	f
EF - hand															n			n	n			n
Rat p9Ka	43	P	S	F	L		G	R	R	T	D	E	A	A	F	Q	K	L	M	N	N	L
Human p9Ka	43	P	S	F	L		G	k	R	T	D	E	A	A	F	Q	K	L	M	s	N	L
Calcyclin	43				t	i	G	s	k	l	q	d	A	e	i	a	r	L	M	e	d	L
Rat S - 100	42	s	h	F	L	e	e	i	k	e	q		e	v	v	d	K	v	M	e	t	L
Rat intestinal	32	P	n	l	L	k	a	s	s	T	l	d					n	L	f	e	e	L
Pig P11	40	P	g	F	L	e	n	q	k	d	p	l	A		v	d	K	i	M	K	d	L
EF - hand			O		O		O	G	0	n	O			O	n			n	n			n
Rat p9Ka	63	D	S	N	R	D	N	E	V	D	F	Q	E	Y	C	V	F	L	S	C		I
Human p9Ka	63	D	S	N	R	D	N	E	V	D	F	Q	E	Y	C	V	F	L	S	C		I
Calcyclin	61	D	r	N	k	D	q	E	V	n	F	Q	E	Y	v	t	F	L	g	a		l
Rat S - 100	62	D	e	d	g	D	g	E	c	D	F	Q	E	f	m	a	F	v	S	m		v
Rat intestinal	49	D	k	N	d	D	g	E	V	s	y	e	E	f	e	V	F	f	k	k		l
Pig P11	60	D	q	c	R	D	g	k	V	g	F	Q		s	f	f	s	L	i	a	g	l
Rat p9Ka	83	A	M	M	C	N	E	F	F	E	G	C	P	D	K	E	P	R	K	K		
Human p9Ka	83	A	M	M	C	N	E	F	F	E	G	f	P	D	K	q	P	R	K	K		
Calcyclin	81	A	l	i	y	N	E	a	l	k	G											
Rat S - 100	82	t	t	a	C	h	E	F	F	E	h	e										
Rat intestinal	69	s	q																			
Pig P11	80	t	i	a	C	N	d	y	F	v	v	h	m	k	q	k	g	k				

Figure 4. The amino acid sequence of rat and human p9Ka and some closely-related calcium-binding proteins of the S-100 family. The amino acid sequences of p9Ka derived from the coding region of the rat [20] and human [51] p9Ka genes, hamster calcyclin [27], rat S-100 protein [25], rat vitamin-D-dependent intestinal calcium-binding protein [24], and porcine P11 protein [28] are aligned for maximum similarity. EF hand refers to consensus amino acid sequences for the variant S-100 EF-hand (amino acids 12–41 of p9Ka) and the non-variant EF-hand (amino acids 55–79 of p9Ka). n, any hydrophobic amino acid; 0, co-ordination position for a calcium ion; E, F, G, K, L, N, particular amino acid residues using the one-letter code, upper case signifying identity with rat p9Ka [20], and lower case signifying non-identity. Bold letters highlight the nine amino acid differences between human and rat p9Ka. The underlined regions of the rat p9Ka sequence indicate peptides of the natural p9Ka protein for which amino acid sequence data is available.

BIOCHEMICAL AND IMMUNOFLUORESCENT SUBCELLULAR LOCALISATION OF p9Ka IN CULTURED CELLS

The location of p9Ka in cells has been studied by examining its distribution in subcellular fractions of p9Ka-expressing cultured cells. When proteins extracted from biochemically-isolated subcellular fractions are subjected to two-dimensional polyacrylamide gel electrophoresis [4], p9Ka is found both in membrane/ cytoskeletal/ microsomal pellet fractions and in the soluble 100 000 g(av) supernatant [44]. In some experiments, the protein spot corresponding to p9Ka splits into two separate, but closely-spaced, isoelectric focusing variants that are present in approximately equal amounts in unfractionated extracts from the cultured cells. The more acidic variant predominates in the membrane/ cytoskeletal / microsomal fractions, whereas the more basic variant predominates in the soluble fraction. These results suggest that a proportion of the p9Ka in these cells may be modified in some, as yet unidentified manner, and that the modified form has a subcellular location which differs from that of the unmodified form [44].

It has been suggested that p9Ka affects the state of tubulin polymerisation in melanoma cells [45], that it interacts with the microfilamental cytoskeleton in rat embryo fibroblasts [46], or that it interacts specifically with non-muscle tropomyosin *in vitro*

[47]. In order to clarify the location of p9Ka in the cultured metastatic mammary cells, the antiserum to p9Ka was used in immunofluorescent experiments.

The benign epithelial cell line, Rama 37, used as the recipient for the DNA in the transfection experiments described above, contains immunofluorescently undetectable levels of p9Ka [21, 48]. The metastatic epithelial cells arising from transfection of Rama 37 cells with the p9Ka gene possessed strongly-staining cytoplasmic filaments which are concentrated in the perinuclear region of the cell (Figure 2a); the staining was completely abolished by prior incubation of the antiserum with rp9Ka [21]. The pattern of immunofluorescent staining obtained with the unblocked p9Ka antiserum was identical to that observed with phalloidin (Figure 2b), which is thought to form a complex with F-actin [49]. These results suggest that in those cells in which it is expressed at a high level, p9Ka associates with a cytoskeletal structure which resembles the actin / myosin, but not the tubulin cytoskeleton. However, in these cells, it is not yet possible to distinguish between a direct interaction with actin [46] or with the non-muscle tropomyosin components of these filaments [47]. So far we have been unable to detect p9Ka in the extracellular medium of metastatic or non-metastatic rat mammary cells as reported for cultures of some bovine fibroblasts and smooth muscle cells [50]. The widespread, cell type-specific distribution of p9Ka, its presence both inside and outside the cell, and its variously reported interactions with calcium ions and microfilamental systems or associated proteins, points to a highly sophisticated role for p9Ka in the area of cell–cell interaction and cell motility.

p9Ka is absent in rat mammary epithelial cells *in vivo* and in cultured cells derived from normal rat mammary glands and benign mammary tumours [4, 11]. However, overexpression of p9Ka in such benign rat breast cells can induce the metastatic phenotype [21, 48]. The primary structure of p9Ka is 91% conserved between rat and man [51] (Figure 4), suggesting that p9Ka may play a similar role in human as in rodent cells.

p9Ka IN HUMAN BREAST CANCER?

The gene for p9Ka has been mapped to human chromosome 1 where it forms part of a multigene family of calcium-binding proteins [52, 53]. From cytogenetic evidence, the commonest abnormalities in breast cancer are those that involve chromosome 1 [54], and such changes include both over-representation of the entire chromosome and translocations of fragments of the chromosome, both of which may lead to increased expression of involved genes [55]. It is likely that the wide occurrence of abnormalities of chromosome 1 represent progressive rather than initiative events in the development of breast cancer. Such changes would be expected to be characteristic of a metastasis-inducing gene, such as p9Ka. Furthermore, p9Ka is located in a region of chromosome 1, q21 [52, 53], that contains a previously-identified fragile site [56]. It is possible that such gross changes in the distal region of chromosome 1 in some human cancer cells may lead to the over-expression of p9Ka, which may in turn contribute to the induction of the metastatic phenotype in human breast cancers. To test this idea, normal and benign human breast cells have been compared with metastatic breast cancer cells for their level of the mRNA for p9Ka. Epithelial cell lines derived from normal mammary tissue immortalised with SV-40 virus [57], or derived from benign human mammary lesions [58] contain only low levels of the mRNA for p9Ka. However, some cell lines derived from malignant metastatic human mammary carcinomas express the mRNA for p9Ka at a higher level (Lloyd and Barraclough, unpublished observations). In a preliminary series of specimens from benign and malignant human lesions, the mean level of the expression of p9Ka mRNA in malignant tumours is statistically significantly higher than in benign lesions (Lloyd and Barraclough, unpublished observations), raising the possibility that, as in the rat, elevated level of p9Ka in human carcinomas is associated with the metastatic phenotype. Further work is now required to identify those cells within the carcinoma which are producing the mRNA for p9Ka. The production of p9Ka in the carcinoma cells raises the possibility that p9Ka is not only a marker of the metastatic phenotype in human breast cancers, but is also a metastasis-inducing protein, and as such, a target for future preventative therapy.

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