REVIEW ARTICLE

Cellular consequences of thrombin-receptor activation

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1. INTRODUCTION

To say that thrombin is a multi-functional protein is rather to understate the case. It is the key enzyme involved in haemostasis, playing important roles at all levels of complexity. First, in the coagulation cascade, thrombin converts fibrinogen into fibrin, which is readily cross-linked to form a clot (reviewed in [1,2]); secondly, thrombin activates blood platelets, causing aggregation and secretion [3,4]; and thirdly, thrombin can elicit mitogenic responses from vascular smooth-muscle cells [5,6]. This latter property is probably most significant in the renewal of damaged blood-vessel walls. Additionally, thrombin is able to elicit responses from cell types as diverse as macrophages [7], monocytes [8] and neutrophils [9]. Perhaps more surprisingly, it is able to regulate neurite outgrowth from cells of neuronal origin [10] and initiate resorption of bone cells [11].

All of these properties of thrombin appear to rely on its action as a serine proteinase, since modification (either chemically [12–14] or mutationally [15–17]) which destroys its proteolytic activity leads to a loss of biological activity. These crucial findings have been explained to a large extent by the recent elegant characterization of a novel, widely expressed thrombin receptor which is activated by proteolytic cleavage rather than by ligand (protein) binding [18,19]. At the same time as elucidation of the mechanism of thrombin signalling there has been a dramatic increase in our understanding of how thrombin signals are mediated within the cell. This therefore seems an appropriate time to assess our current knowledge and perhaps to try to predict areas where the greatest advances will be made in the immediate future. In this review, because of limitations in length, emphasis will be placed on recent advances, in particular on the characterization of the thrombin receptor and in the mechanisms of intracellular signalling. Whilst most studies of thrombin have concentrated on its action on cells involved in blood clotting and wound healing, it is now becoming apparent that it can modulate the growth and differentiation status of cells of neuronal origin. A consideration of recent advances in this area of study will form the third major theme of this review.

2. STRUCTURE OF THROMBIN

Thrombin is generated in circulating plasma by the cleavage of prothrombin, when it forms part of the prothrombinase complex. The other components of this complex, which are essential for the proteolytic reaction, are activated Factors X and V, Ca^{2+} and membrane phospholipid (reviewed, for example, in [1]). Prothrombin itself is synthesized in the liver and represents a unique class of vitamin K-dependent zymogens in that it has two 'kringle domains' of approximately eight amino acids each and an N-terminal 'Gla domain' containing ten γ-carboxyglutamic acid (Gla) residues [1,20] (Figure 1a). Conversion of prothrombin into thrombin involves the cleavage of peptide bonds C-terminal to Arg^{271} and Arg^{320}. The N-terminal region of prothrombin containing the Gla and kringle domains is inactive and appears to have no further biological role [21]. Thus thrombin is about half the size of prothrombin (39 kDa compared with 71.6 kDa) and comprises an A (light) chain (49 residues) and a B (heavy or catalytic) chain (259 residues) joined by a disulphide link between Cys^{22} (A chain) and Cys^{119} (B chain) (throughout this review the N-terminal amino acid of either prothrombin or the A and B chains of thrombin is designated residue 1 of the respective protein, as in [20] and Figure 1a). Further cleavage of the thrombin A chain can occur, removing a further 13 amino acids. Three carbohydrate moieties are present on each prothrombin molecule, with one being present in thrombin (linked to Asn^{53} on the B chain).

The amino acid sequence similarities between thrombin and other serine proteinases such as trypsin encouraged a number of structural predictions to be made. More recently, a high-resolution X-ray-crystallographic study of thrombin bound to various small molecules has allowed the structure of human and bovine thrombin to be determined at high resolution (see, for example, [22–24]). These studies have been reviewed in considerable detail by Stubbs and Bode [25], and readers in need of a detailed account of thrombin structure are referred to that excellent article. It is, however, worth briefly describing here a few of the major structural features of the molecule so that its mode of action can be better understood. Thrombin is a highly structured globular protein and is ellipsoid in shape. A deep narrow groove containing the active site runs across the molecule with the three catalytic amino acids, His^{43}, Asp^{99} and Ser^{205}, lying at its base (Figure 1b). The short A chain is held in place on the side of the protein opposite to that of the active site. Until the cloning of the thrombin receptor, the primary natural substrates for thrombin had been considered to be the A and B chains of fibrinogen, although hydrolysis of peptide bonds in a number of other proteins occurs *in vivo*. In all cases of mammalian proteins thrombin cleaves a peptide bond C-terminal to an Arg residue. A number of amino acids can occupy the P1′ position (C-terminal to the Arg), but they are generally small and hydrophilic, such as glycine in the fibrinogen A and B β chains or serine in the thrombin receptor, Factor V and Factor VIII. The P2 position (N-terminal to the Arg) is usually occupied by a Pro, but not invariably so. Restrictions can also be seen in the amino acids allowed for P3 (usually hydrophilic and/or small) and P4 (usually large and hydrophobic) in thrombin substrates. The much greater substrate specificity shown by thrombin over other serine proteinases is probably attributable to the depth and narrowness of the active-site cleft, making the active-site amino acids much less accessible to protein substrates in general (Figure 1b).

A second important structural feature involved in the interaction of thrombin with its substrates is the anion-binding exosite (basic patch), which is centred around the loop between Lys^{65} and Lys^{77}. Thus a series of basic amino acids (Arg^{68}, Arg^{70} and Arg^{73}) are in close proximity to Arg^{20}, Lys^{21} and Lys^{154} in the

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Figure 1 The structure of thrombin

(**a**) The amino acid sequence of preprothrombin is shown. The peptide bond at the C-terminus of the preproleader peptide is cleaved by a signal peptidase, and the bonds required for conversion of prothrombin to thrombin are cleaved by Factor Xa. The positions of 13 introns (A–M) in the prothrombin gene sequence are indicated, as are the Gla and kringle domains. The active-site amino acids His^{43}, Asp^{99} and Ser^{205} are indicated. Asn^{76} and Asn^{100} in prothrombin and Asn^{53} in thrombin are sites of glycosylation (◆). Amino acid numbering is as follows: preproleader sequence −43 to −1; prothrombin fragment 1, 1–271; thrombin light (A) chain, 1–49; thrombin heavy (B) chain, 1–259. Reproduced from Friezner, Degen and Davie [20] with the permission of the publishers of *Biochemistry*. (**b**). The three-dimensional structure of the thrombin heavy chain. The ribbon structure of the heavy chain of human thrombin is shown (from the P1DWE PDB entry). The positions of the active-site amino acids (His^{43}, Asp^{99} and Ser^{205}) and the basic patch amino acids (Arg^{20}, Lys^{21}, Arg^{68}, Arg^{70}, Arg^{73}, Lys^{106} and Lys^{107}) are shown, as is the position of the active-site cleft.

folded protein, with Lys[77], Lys[106] and Lys[107] lying somewhat further away (Figure 1b). These residues give rise to a positively charged patch which forms a site of interaction with acidic areas on fibrinogen, fibrin, thrombomodulin, hirudin and the thrombin receptor. The interaction of thrombin with its receptor is discussed in more detail in section 3 below.

3. STRUCTURE AND MODE OF ACTION OF THE THROMBIN RECEPTOR

(i) Structure of the mammalian thrombin receptor

The ability of thrombin to elicit cellular responses has been well documented for many years. During that time a number of thrombin-binding proteins were identified [26], but none would initiate a second-messenger response and was therefore unlikely to represent a receptor molecule. Many of these inconsistencies and anomalies were resolved when thrombin receptors from human platelets [18,19], hamster lung fibroblasts [27], rat aortic smooth-muscle cells [28] and, later, *Xenopus laevis* [29], were cloned and sequenced. Largely on the basis of the derived amino acid sequences of these proteins a model was proposed for the mechanism of receptor activation which has stood up well to experimental analysis.

The human thrombin receptor comprises 425 amino acids and has a number of structural features in common with the classical G-protein-linked receptors (Figure 2), being most similar to receptors for neuropeptides and glycoprotein hormones [18]. Seven helical hydrophobic transmembrane regions have been proposed [18] giving rise to three intra- and three extra-cellular loops and a C-terminal intracellular tail and a long N-terminal extracellular domain (Figure 2). Within this latter region are a number of structural determinants which are essential for the correct functioning of the receptor. A thrombin cleavage site [LDPR↓SFLL (one-letter amino acid code)] similar to that present in Protein C [18] is present between residues 41 and 42 (in the human protein). Two thrombin interaction sites have been proposed on the receptor: one immediately N-terminal to Arg[41] is involved in recognition of the cleavage site, whilst the second, between residues 53 and 64, shows sequence similarity to a C-terminal region of hirudin and is involved in interaction with the thrombin anion-binding exosite ([23] and Figure 1b). Both of these sites are highly conserved between the cloned mammalian thrombin receptors, but rather less so for the *Xenopus* protein [29] (Figure 3).

Three-dimensional structural studies of the thrombin receptor have been limited by the lack of sufficient protein, and therefore use has been made of the peptide-mimetic approach. Thus an examination of the structure of peptides identical with parts of the N-terminal extracellular domain has allowed the identification of overlapping turns in the region between residues 47 and 51 stabilized by a hydrogen bond 48_{CO}–51_{NH} [30] as well as a 3_{10} helix covering the seven residues between Pro[40] and Arg[46]. This latter structural motif is stabilized by a charged hydrogen bond between the side chains of Asp[39] and Arg[41] [30]. Obviously, an element of doubt exists as to whether such structures, based on short synthetic peptides, exist in the intact protein, although computer modelling has indicated that it is possible to fit the proposed peptide structure into the substrate-binding cleft of thrombin (see below).

NMR spectroscopic and crystallographic studies of synthetic receptor peptides in the presence of thrombin have allowed the sites of interaction to be closely defined. In the bound state a receptor peptide comprising amino acids Leu[38]–Glu[60] adopts an S-shaped conformation with three anti-parallel strands (Leu[38]–Pro[40], Arg[41]–Leu[45] and Asp[50]–Pro[54]) about 0.7 nm (7 Å) apart [31]. These data are reasonably compatible with the helical structures between Pro[40] and Arg[46] and turns around Asn[47]–Lys[51] reported by Smith et al. [30]. The three strands of the receptor peptide appear to correspond to the thrombin cleavage site, the agonist peptide and the hirudin-like domain. It should be noted, however, that in the crystallographic studies reported by Mathews et al. [31], each receptor peptide binds to two adjacent thrombin molecules, one contributing to the catalytic binding site and the second to the anion-binding exosite. So far there is no evidence for such a structure existing *in vivo*. Interaction of the receptor with the thrombin catalytic (active centre) site primarily involves the few residues on the receptor immediately N-terminal to the cleavage site (Leu[38]–Arg[41]) [31,32]. In thrombin-receptor peptide crystals the side chain of Leu[38] in the receptor occupies a hydrophobic site formed by Ile[179] and Trp[227] of thrombin, with a hydrogen bond being formed between the Leu backbone and Tyr[37] [31,32]. Perhaps more importantly, a salt bridge is formed between the receptor Asp[39] and His[43] in the thrombin catalytic site (Figure 1b). A hydrogen bond has also been demonstrated between Asp[39] or Pro[40] and thrombin Gly[230] [31,32], orienting Asp[39] towards Arg[233] of thrombin and reducing the mobility of the receptor cleavage site. Interaction of receptor peptides at the anion-binding exosite of thrombin appears to involve primarily the D[50]KYEPF[55] motif in a manner analogous to that seen for the hirudin homologues hirugen and hirulog 1 in complexes with thrombin [33]. Additional evidence for the receptor KYEPF sequence being the primary area of interaction with the anion-binding exosite is provided by the observations that substitutions for Tyr[52], Glu[53] and Phe[55] in the receptor result in loss of receptor response to thrombin [19] and that appropriate synthetic receptor peptides can displace hirudin from the exosite [34].

Thus it seems probable that, in the interaction of thrombin with its receptor, an initial contact is made between the anion-binding exosite and the receptor sequence KYEPF. In the second step the orientated receptor peptide binds to the active site with receptor Leu and Pro interacting with the apolar pocket. It has been proposed that, before cleavage, the helical structure around the cleavage site must be unwound, disrupting the hydrogen bond between Asp and Arg, thus freeing the Arg residue [30] and allowing it to move into the specificity pocket. Cleavage of the receptor then occurs, generating a new N-terminal sequence which is free to trigger the intracellular response. It is believed that this occurs by interaction of the first five or six amino acids with a binding site possibly located on extracellular loop 2 (amino acids 244–268) and/or the N-terminal exodomain (amino acids 76–93) [29,35] (Figure 2).

(ii) Thrombin-receptor-activating peptides (TRAPs)

One of the most telling pieces of evidence in favour of the proposed model for receptor activation has been the observation that short peptides identical with the sequence C-terminal to the receptor cleavage site (Arg[41]↓Ser[42]) can duplicate the actions of thrombin. Thus TRAPs can cause platelet aggregation [18,36–42], release of intracellular Ca^{2+} stores [38,42–44], 5-hydroxytryptamine release [36], adenylate cyclase inhibition [38,45,46], stimulation of DNA synthesis and mitogenesis [46,47], neurite retraction [48], activation of MAP (mitogen-activated protein) kinase [49,50] and a number of other effects associated with thrombin stimulation. On the basis of these studies, it is possible to define quite precisely the structural determinants within the peptides (and therefore in the N-terminal region of the receptor) which are essential for receptor activation. Thus substitutions or 'deletions' outside the amino acid sequence SFLLRN (residues

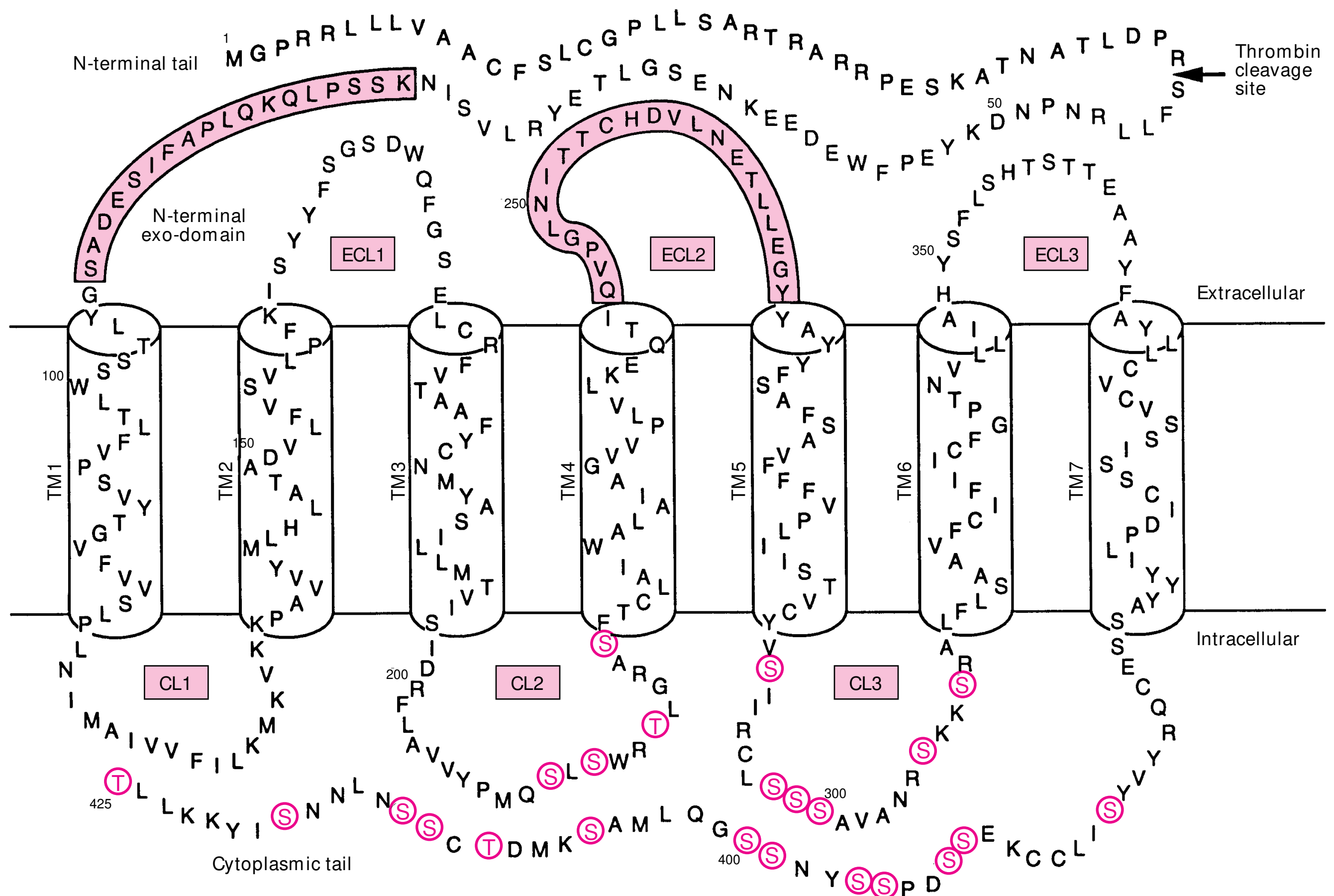

Figure 2 Structure of the human thrombin receptor

The amino acid sequence is arranged through the membrane, as suggested by Vu et al. [18] and Gerszten et al. [29]. The thrombin cleavage site (←), extracellular domain, extracellular loops (ECL) and cytoplasmic loops (CL) are indicated. Potential intracellular phosphorylation sites (encircled red letters) and amino acid residue numbers are marked. The extracellular domains considered to be the sites of interaction with the N-terminal receptor peptide are indicated by boxed-in stretches of amino acids shaded pink.

		Cleaveage-site recognition domain (P_4 P_3 P_2 P_1)	↓ TRAP domain (P_1')		Interaction domain with the thrombin anion-binding exosite	
Human	T N A T	L D P R	S F L L R N	P N D	K - Y E P F	- W E D
Mouse	T D A T	V N P R	S F F L R N	P S E	N T F E L V	P L G D
Rat	P Y A T	P N P R	S F F L R N	P S E	D T F E Q F	P L G D
Hamster	T D A T	V N P R	S F F L R N	P G E	N T F E L I	P L G D
Frog	H S N N	M T I K	T F R I F D	D S E	S E F E E I	P W - D

Figure 3 Comparison of the amino acid sequences of part of the extracellular N-terminal domain of vertebrate thrombin receptors

Sequences have been aligned for maximum identity. The thrombin cleavage site (↓), the cleavage recognition domain, the hirudin-like domain which interacts with the thrombin anion-binding exosite and the receptor N-terminal domain (TRAP) are all identified. – indicates a gap in the sequence introduced to allow for better alignment. References for the sequences are given in the text.

1–6 of the cleaved receptor) appear to make very little difference to the biological activity of the peptide [36–39] and even deletion of amino acid 6 (Asn) reduces the potency of the peptide only marginally [36,37,39]. Within the region Ser–Arg, however, only very conservative replacements are allowed. A free N-terminal amino group is essential for activity, *N*-acetyl derivatives being unable to elicit a response from platelets [36,37,40]. There is a requirement for a small, neutral hydrophilic amino acid at the N-

terminus, such that Gly, Ala, Thr, Cys and Ile can substitute for Ser with only limited loss of activity [36–38,40,46]. There appears to be an absolute requirement for an aromatic residue at position 2, with even the conservative Tyr-for-Phe substitution causing a reduction in activity [41,46]. Replacement of the Leu residues at positions 3 and 4 with Ala also results in a loss of the peptide's ability to cause platelet aggregation and 5-hydroxytryptamine release [37,41], although substitution at position 3 appears to have a somewhat more deleterious effect on the activity [36,38]. Similarly, considerable constraints exist on the amino acid present at position 5 in the peptide, there being a requirement for a basic residue for the retention of activity [36,41,46].

NMR-spectroscopic studies carried out on synthetic peptides covering the N-terminus of the receptor exposed after thrombin cleavage indicate little secondary structure attributable to the first five amino acids [30]. Thus it seems likely that recognition of the tethered ligand by other region(s) of the receptor probably involves 'docking' of the relatively unstructured and possibly extended peptide into a pocket where side-chain recognition is all important.

The amino acid sequences of mammalian thrombin receptors are conserved in the proposed thrombin-binding sites (both the region which binds to the thrombin active centre and that which is recognized by the anion-binding exosite) and in the sequence of the tethered ligand exposed after cleavage. The only difference in this latter area is the substitution of Phe for Leu at position 3 in all the rodent sequences (Figure 3). This substitution does not appear to impart a species specificity, as the weight of evidence suggests that there is no difference in the ability of the rodent or human peptides to elicit a response (in either human or rodent cells) [46,51]. It should be noted, however, that, in at least one recent study [52], rabbit and rat platelets did not respond fully to the human TRAP. The amino acid sequence of the *Xenopus* receptor is quite different to the mammalian protein and is unresponsive to human peptides [29]. This is probably a result of substitution in the as-yet-unidentified ligand-binding site. The two major changes in the frog tethered ligand are Arg for Leu at position 3 and Phe at position 5 (Figure 3). As expected, this peptide is unable to activate the human receptor [29].

(iii) Different responses to thrombin and TRAPS

Not surprisingly, differences have been observed in the concentrations of thrombin and receptor peptides required to elicit a similar level of response (see, for example, [46]). Naïvely, this may be explained on the basis of either very high local concentrations of tethered ligand present after thrombin stimulation, or a more appropriate orientation adopted by the tethered peptide compared with one free in solution. Perhaps more interestingly, different cellular responses to receptor activation by thrombin and by peptides have been observed. For example, whilst thrombin is mitogenic for hamster lung fibroblasts, the synthetic N-terminal peptide is not. Treatment with growth factors (fibroblast growth factor) in addition to peptide is required for re-entry into the cell cycle [49]. This observation has been explained by the ability of thrombin to cause sustained activation of MAP kinase, whilst stimulation with the peptide only gives a transient increase in activity [49]. Additionally, differences in intracellular Ca^{2+} signals in osteosarcoma-like cells have been reported in response to thrombin and a thrombin-receptor-agonist peptide (the former inducing a transient increase in $[Ca^{2+}]$, whilst the latter produced a biphasic response [17]). Even in the human platelet system, different responses to thrombin and receptors peptides have been seen. Thus the extent of aggregation, activation and association of the functional integrin glycoprotein IIb-IIIa and Src with the cytoskeleton, phosphatidate production, arachidonic acid release and phosphorylation of phospholipase A_2 (PLA_2) (see section 4) is always greater following thrombin stimulation [53]. These sorts of data have been variously interpreted as evidence for the existence of one or more thrombin receptors in addition to the cloned molecule or for the inability of peptides to mimic completely the action of thrombin due to signals which may be generated by interaction of the intact protein with the receptor further to those produced by proteolysis [49,52,54,55].

It has long been acknowledged that thrombin can produce graded responses in a number of different cell types [56,57]. How this might be achieved through a receptor activated only by a single proteolytic step has proved a matter of some debate, since it might be expected that all receptor extracellular domains would eventually be cleaved, even by very small amounts of thrombin. However, it has recently been demonstrated that total phosphoinositide hydrolysis over a particular time interval correlated well with the total extent of receptor cleavage [58]. It has therefore been suggested that stimulation of a receptor molecule generates a single release ('quantum') of second messenger before becoming inactive. Thus the cellular response to thrombin is regulated by the number of quanta released (i.e. the concentration) and the subsequent rate of second-messenger breakdown (removal), which presumably stays constant [58].

Support for the view that the receptor is capable of considerable subtlety has been provided by an examination of the effects of prothrombin on neuronal cells, where it has been shown that even though responses are only triggered after conversion into thrombin (and then presumably stimulation of the thrombin receptor), prothrombin will not duplicate all of the biochemical events observed after thrombin treatment [59]. It has been concluded that this is due to the fact that the concentration of prothrombin-generated thrombin at the cell surface never reaches that found after addition of the purified protein and is therefore not able to generate the full range of intracellular signals [59].

(iv) Thrombin-receptor inactivation and cellular processing

It has been recognized for some time that desensitization of the thrombin receptor requires the activity of an intracellular kinase [60], but it has only recently been directly demonstrated that rapid phosphorylation of serine or threonine residues in the cytoplasmic tail occurs very soon after thrombin or peptide stimulation and that this correlates well with receptor inactivation [61] (Figure 2). Phosphorylation is probably attributable to a G-protein-coupled receptor kinase [61] and not protein kinase C (PKC) [61,62]. Differences in susceptibility of the receptor to inactivating phosphorylation could explain the differences between thrombin and peptide activation. If, for example, the cleavage step, rather than ligand binding, is more important in inducing a conformational change allowing receptor phosphorylation and desensitization, a more prolonged response might be expected after peptide stimulation [17]. This would not, of course, explain the more transitory effects observed in some systems following peptide activation [49]. Although a specific thrombin-receptor kinase has not yet been identified, the observation that the β-adrenergic receptor kinase 2 (BARK2) is much more active than BARK1 or the rhodospin kinase (all kinases involved in the down-regulation of G-protein-linked receptors [62]) lends weight to the notion that such a specific kinase could be present in the cytoplasm of thrombin-sensitive cells. The well-characterized G-protein-coupled receptor kinases appear to interact with the activated receptors, either through the cytoplasmic loops or the C-terminal tail, although it should be

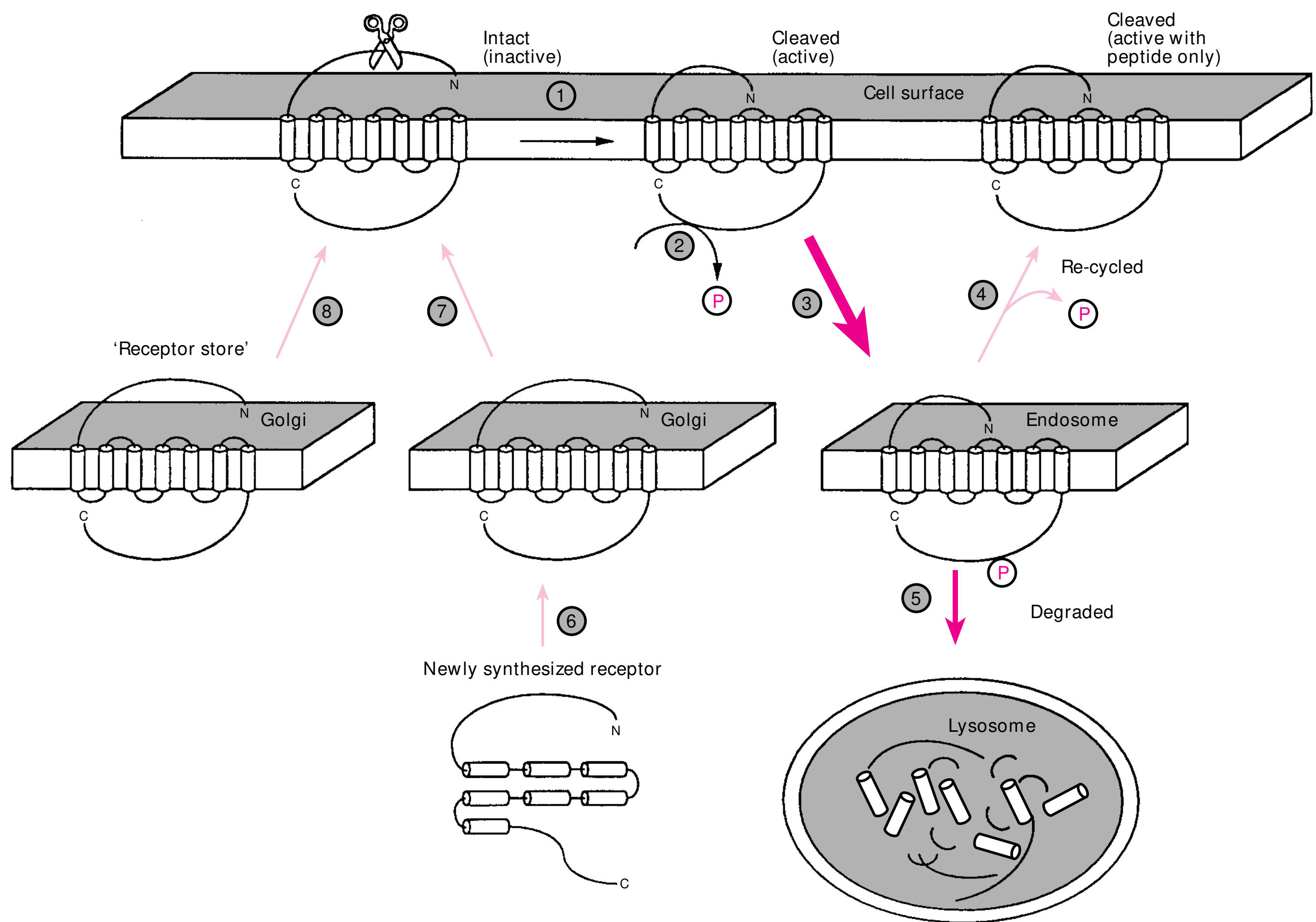

Figure 4 Thrombin-receptor trafficking

Surface thrombin receptors are normally activated by proteolytic cleavage by thrombin (1), and after an appropriate time are inactivated by phosphorylation by a receptor kinase (2). Internalization (heavy red arrows) occurs via coated pits and endosomes (3). Receptor can then be either recycled (4) to the surface, possibly following dephosphorylation (where it can only be activated by TRAP), or degraded in the lysosomes (5). Newly synthesized receptor (6) is processed in the Golgi and transported to the surface (7), but additional molecules are kept in a 'receptor store' ready for rapid activation (8), thus restoring the cells' responsiveness to thrombin after an initial stimulation. This scheme is based on models proposed by Coughlin and co-workers and Brass and co-workers [63–65].

noted that the sites of phosphorylation probably reside in this latter part of the molecule (reviewed in [62]) (Figure 2). These observations are consistent with the reported properties of the thrombin-receptor kinases [61].

A second mechanism for down-regulation of the thrombin receptor following activation has been identified. Soon after (within 1 min) thrombin stimulation of HEL and CHRF cells, most of the receptor molecules appear to be internalized in coated pits in the plasma membrane [63] (Figure 4). Over the course of about 30 min receptors can be detected in, sequentially, endosomes, prelysosomes and mature lysosomes [63,64]. In some cell types (e.g. HELs) considerable time needs to elapse between receptor activation (and internalization) and the regeneration of thrombin-sensitivity [63,65]. This correlates well with the *de novo* synthesis of receptors and is blocked by protein-synthesis inhibitors [63,64]. However, in cells of endothelial origin and fibroblasts, a population of thrombin receptors is present within the cell (but not on the surface), possibly located in the Golgi [64] (Figure 4). Thus activation of the surface thrombin receptor initiates transport of these intracellular molecules to the cell surface, where they replace cleaved molecules and are then ready to allow the cell to respond to a second round of thrombin stimulation. It seems likely that this ability distinguishes endothelial cells, which may need to respond to thrombin repeatedly, from platelets, which generally are required to make a single response (in the form of granule release and aggregation) [64].

After internalization, most the thrombin-receptor molecules are degraded in lysosomes. However, in CHRF and HEL cells a proportion are recycled to the cell surface over a period of 1–3 h [16]. If the cell has been stimulated with receptor peptide, the recycled receptors are sensitive to either thrombin or additional peptide (presumably because an inactivating phosphorylation step has not taken place). If the cells have been stimulated with thrombin, however, they are only responsive to receptor peptide. At a later time, full responsiveness to thrombin stimulation returns as new receptor molecules are synthesized (Figure 4).

It is important to remember that considerable differences in thrombin-receptor internalization and trafficking have been observed in different cell types. As noted above, the data obtained from a study of endothelial cells does not duplicate that from the HEL and CHRF288 cell lines [16,63–65]. Additionally, recent evidence suggests that receptor internalization in platelets occurs to only a very limited extent [65,66].

(v) The contribution of growth factors to thrombin activity

Whilst stimulation of the thrombin receptor can, in many cell types, induce mitogenesis, in some circumstances additional ligands are required for cell growth. For example, there is a synergistic effect produced by thrombin and various growth factors [epidermal growth factor, platelet-derived growth factor and basic fibroblast growth factor (bFGF)] on the proliferation of hamster fibroblasts [67], human vascular endothelial cells [68,69] and vascular smooth-muscle cells [70]. It has been suggested that these growth factors may activate tyrosine kinases essential for mitogenesis [71], thus complementing the action of thrombin. Additionally, it is possible that thrombin may elicit an autocrine response from cells, namely triggering release of growth factors which can then activate surface receptors [70]. The weight of evidence, however, supports the view that, for most cell types, there is no requirement for ligands in addition to thrombin to develop the full range of responses.

(vi) Other thrombin receptors

Whilst the cloned receptor described here is now accepted as the major, if perhaps not the only, receptor, a body of evidence exists for additional molecules responsive to thrombin on the surface of cells. These so called 'high-affinity sites' are activated by thrombin binding rather than by proteolysis (reviewed, for example, in [26,72]). It has been suggested that peptides equivalent to regions of thrombin can bind to the cell surface and initiate cellular responses such as mitogenesis with no requirement for proteolytic activity [73].

4. INTRACELLULAR RESPONSES TO THROMBIN-RECEPTOR ACTIVATION

The thrombin receptor stimulates a number of phospholipid-directed enzymes, generating both lipid-soluble and water-soluble second messengers. Although originally identified as a member of the G-protein-coupled receptor family, studies with specific inhibitors of tyrosine kinases and monomeric, low-molecular-mass G-proteins suggest that these molecules also participate in thrombin-stimulated phospholipid metabolism. Similarly, thrombin-stimulated MAP kinase activation is dependent on heterotrimeric G-proteins, tyrosine kinases and low-molecular-mass G-proteins (summarized in Figure 5). However, the precise relationship between G-protein activation, tyrosine kinase activation and Ras activation remains ill-defined.

Since gross morphological changes accompany thrombin-mediated mitogenesis and differentiation, thrombin must also regulate actin cytoskeleton assembly and disassembly. The ability of the thrombin receptor to initiate changes in cytoskeletal reorganization defines more subtle roles for thrombin in controlling physiological processes in terminally differentiated cells, for example thrombin-stimulated aggregation and granular release in platelets [74], thrombin-stimulated cell motility [75] and thrombin-stimulated neurite retraction [10]. The roles of polyphosphoinositides and Ca^{2+}, heterotrimeric G-proteins, tyrosine kinases and monomeric G-proteins in the control of cytoskeletal dynamics have recently been comprehensively covered in two outstanding reviews [76,77].

(i) Thrombin stimulation of phosphoinositidase C (PIC) activity

(a) Role of heterotrimeric G-proteins

Many receptors possessing seven transmembrane domains, including the thrombin receptor, couple through heterotrimeric G-proteins to stimulate a PICβ activity, directed towards PtdIns(4,5)P_2, generating the second-messenger molecules Ins(1,4,5)P_3 and diacylglycerol (DAG) (reviewed in [78]). PICβ1 and PICβ4 are regulated predominantly by GTP-ligated Gα subunits of the G_q subfamily, whereas PICβ2 and PICβ3 are more responsive to G$\beta\gamma$ subunits derived predominantly from G_i (reviewed in [79]). Agonists which couple to G_i are sensitive to inhibition by pertussis toxin, which inhibits G_i function specifically by ADP-ribosylating Gα_i at a Cys residue four amino acids from its C-terminus [80]. G_q, G_{12} and G_s lack this critical Cys residue and are therefore unaffected by the reagent [81–83].

Thrombin stimulation of PIC activity in platelets [84], HEL cells [85], Chinese-hamster CCL39 cells [86], chick embryonic heart cells [87] and vascular smooth-muscle cells [88] is sensitive to pretreatment with pertussis toxin, suggesting a role for G$\beta\gamma$ subunits derived from G_i in stimulating PIC activity in these cells. In contrast, thrombin-stimulated PIC activity in IIC9 Chinese-hamster embryonic fibroblasts [89], 3T3-fibroblasts [90], VMR 106-H5 osteosarcoma cells [91] and human umbilical-vein endothelial cells [92] is pertussis-toxin-insensitive, potentially implicating members of the G_q subfamily in stimulating PIC activity. The role of Gα subunits in thrombin-responsive cells has been studied by microinjecting inhibitory anti-α_q, anti-α_o and anti-α_i monoclonal antibodies into CCL39 cells and analysing their effects on TRAP-induced Ca^{2+} mobilization and DNA synthesis [93]. Both anti-α_q and anti-α_o monoclonal antibodies significantly inhibit intracellular Ca^{2+} release and DNA synthesis, whilst anti-α_i monoclonal antibodies have no effect, implicating α_q and α_o in thrombin-stimulated mitogenesis.

PICβ1 is involved in thrombin-stimulated PtdIns(4,5)P_2 hydrolysis in CCL39 cells. A CCL39 derivative that expresses constitutively low levels of PICβ1 compared with the parental cell line has impaired coupling to effectors in response to thrombin [94]. Thus thrombin-stimulated inositol phosphate production and cytosolic Ca^{2+} mobilization are reduced, particularly when the external Ca^{2+} concentration is low. Moreover, thrombin activation of effectors that lie downstream of PICβ1 activation in these cells, notably phospholipase D (PLD) and cytosolic PLA_2 [see section 4(v)], are also reduced in activity. The overexpression of Gα_q, PICβ1 and PICβ2 in *Xenopus* oocytes significantly enhances thrombin-stimulated Ca^{2+} release, further implicating these molecules in thrombin signalling [95]. Similarly, overexpression of PICδ1 in Chinese-hamster ovary (CHO) cells greatly enhances thrombin-stimulated PtdIns(4,5)P_2 hydrolysis [96]. This response is potentiated by ionomycin in intact cells and guanosine 5′-[γ-thio]triphosphate (GTP[γS]) in permeabilized cells, indicating roles for both G-proteins and Ca^{2+} in coupling thrombin receptor activation to the regulation of PICδ1.

(b) Role of non-receptor protein tyrosine kinases

The thrombin receptor can activate non-receptor tyrosine kinases. Thus thrombin stimulation induces the tyrosine-specific phosphorylation of a large number of proteins [97,98] as judged by Western blotting using tyrosine phosphate-specific antibodies. A few of these polypeptides have been identified, although the identity of the majority remains unknown at present. The best characterized of the substrates are Src and FAK, but recent evidence has indicated that JAK2, implicated in transcriptional regulation, and Syk, found predominantly in haemopoietic cells, are phosphorylated in response to thrombin-receptor activation [97–102].

Thrombin stimulates a rapid and transient increase in the specific activity of Src, followed by the translocation of the activated protein to a cytoskeleton-rich fraction [103]. In the

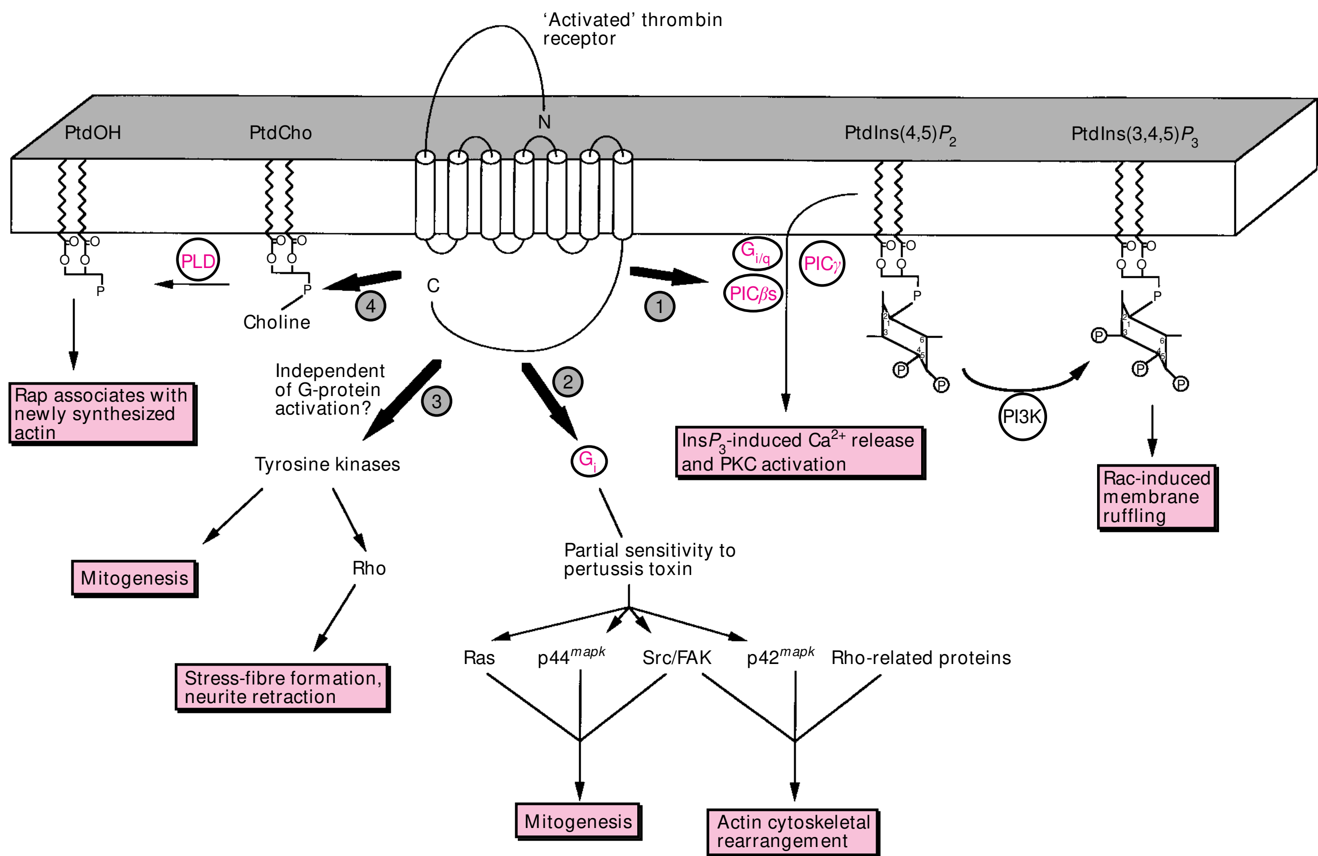

Figure 5 Pictorial depiction of the roles of G-proteins, non-receptor tyrosine kinases and Ras-related proteins in signal transducing events initiated by thrombin receptor activation

(1) PIC and PI3K utilize PtdIns(4,5)P_2 to generate the second messengers InsP_3 and PtdIns(3,4,5)P_3 respectively. (2) Role of G_i in activating other effector molecules, in particular Ras, MAP kinases and tyrosine kinases. (3) Role of tyrosine kinases in thrombin-mediated responses. (4) PLD-catalysed degradation of PtdCho to phosphatidic acid (PtdOH). See the text for a more detailed explanation of the potential physiological roles of the effector molecules that are stimulated by thrombin.

initial 15 s of thrombin stimulation, Src is dephosphorylated on Tyr^{527} and is subsequently phosphorylated at Tyr^{416}, both residues which are thought to be critical in controlling activity [104,105]. Other studies indicate that thrombin-stimulated PKC might be important in stimulating Src activity by directly phosphorylating Ser^{12} [106]. Src activation by thrombin is partially inhibited by pertussis toxin, suggesting that molecules other than G_i may participate in its activation [107].

The tyrosine kinase inhibitor tyrphostin AG-213 has been used to define the role of PICγ in thrombin-stimulated platelets [108]. Thrombin-stimulated inositol phosphate formation, platelet aggregation and 5-hydroxytryptamine secretion were substantially inhibited by tyrphostin AG-213. Thrombin also stimulated the appearance of PICγ in anti-phosphotyrosine immunoprecipitates. This work has recently been extended to demonstrate the tyrosine kinase-dependent recruitment of PICγ1 to the cytoskeleton in thrombin-stimulated platelets [109]. PICγ2 is also a substrate for tyrosine phosphorylation in human platelets in response to thrombin [110]. Src can couple to PICγ to stimulate the tyrosine kinase-dependent hydrolysis of PtdIns(4,5)P_2 [111], though the precise role of Src in thrombin-stimulated PtdIns(4,5)P_2 hydrolysis is not fully understood.

(c) Role of Ras-related proteins

The Ras-related protein Rho, which is integral to mediation of stress-fibre formation [112], is implicated in thrombin-induced responses [see section 4(iii)]. Moreover, it has been suggested that Rho may contribute indirectly to the regulation of thrombin-stimulated PtdIns(4,5)P_2 hydrolysis by stimulating a PtdIns4P 5-kinase activity, thus serving to maintain the levels of agonist-responsive PtdIns(4,5)P_2 [113]. The evidence that Rho, in its GTP-bound form, stimulates PtdIns4P 5-kinase activity in lysates obtained from Swiss-3T3 cells is based on the observation that botulinum C3 exozyme (which specifically ADP-ribosylates Rho in its effector domain, inhibiting its function), inhibits GTP[S]-stimulated PtdIns4P 5-kinase activity. Moreover, lovastatin, which inhibits post-translational acylation and thus membrane targeting of Ras-related proteins, substantially reduces thrombin-stimulated Ca^{2+} mobilization in Swiss 3T3 cells. The botulinum C3 exozyme has a similar effect on thrombin-stimulated Ca^{2+} mobilization when microinjected into C3H10T$\frac{1}{2}$ cells. The reduction in thrombin-stimulated Ca^{2+} mobilization in these circumstances has been suggested to be due to reduced PtdIns4P 5-kinase activity [113].

(ii) Thrombin stimulation of phosphoinositide 3-kinase (PI3K)

(a) Synthesis of 3-phosphorylated inositol lipids

PI3K specifically phosphorylates PtdIns(4,5)P_2, following agonist stimulation [114,115]. Thus far, two agonist-stimulated PI3K isoforms, both with a preferred substrate specificity towards

PtdIns(4,5)P_2, have been identified in cells. The PI3K harnessed predominantly by tyrosine kinases is a heterodimer, consisting of a p85 regulatory subunit and a p110 catalytic subunit [116]. A 215 kDa PI3K activity has also been purified from human neutrophils and is specifically activated by the $\beta\gamma$ subunits of G-proteins [117].

Thrombin stimulates the synthesis of PtdIns(3,4,5)P_3 in platelets [118] by activating a PI3K directed towards PtdIns(4,5)P_2 [119]. Thrombin-stimulated PtdIns(3,4)P_2 formation is due to either PtdIns(3,4,5)P_3 dephosphorylation or PtdIns4P 3-kinase activation [119]. The possibility of a thrombin-activatable PtdIns4P 3-kinase cannot be ignored, since it appears that the major 3-phosphorylated inositol lipid of unstimulated cells, PtdIns3P, is synthesized by a PI3K directed towards PtdIns [120]. Indeed, some reports suggest that thrombin can stimulate PtdIns(3,4)P_2 formation independently of PtdIns(3,4,5)P_3 synthesis [121,122], implying that a PtdIns(4,5)P_2-directed PI3K might not be essential for PtdIns(3,4)P_2 synthesis. Thrombin-stimulated PtdIns(3,4)P_2 synthesis correlates well with tyrosine kinase activation [122].

(b) Regulation of thrombin-stimulated PI3K

Some efforts have been made to determine the regulatory mechanisms underlying thrombin stimulation of PI3K activity in platelets. The Ca^{2+}-ionophore A23187 and phorbol dibutyrate, a potent activator of the Ca^{2+}/DAG-sensitive PKC isoenzymes, were used in an attempt to duplicate thrombin activation of PI3K. However, elevation of intracellular Ca^{2+} or activation of PKC were insufficient to activate PI3K fully [118]. A later report from the same laboratory suggested that protein kinases and G-proteins contributed to the thrombin activation of PI3K; thus staurosporine inhibited the production of the 3-phosphorylated inositol lipids, whilst pertussis toxin inhibited PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 formation by about 25 % [123].

Upon thrombin stimulation of platelets, increased PI3K activity can be detected in anti-phosphotyrosine, anti-Src and anti-Fyn immunoprecipitates [124], implicating tyrosine kinases and p85/p110 PI3K in the thrombin-stimulated synthesis of 3-phosphorylated inositol lipids. Indeed, thrombin can stimulate the translocation of both Src [section 4(i)(b)] and p85/p110 PI3K to the cytoskeleton [125,126]. Tyrosine kinases are further implicated in this process, since the tyrosine kinase inhibitor tyrphostin AG-213 inhibits thrombin-stimulated PtdIns(3,4)P_2 production in platelets by half [108]. Increases in p85 levels have also been detected in anti-phosphotyrosine immunoprecipitates, suggesting that thrombin stimulates either the recruitment of p85 to phosphotyrosine complexes or tyrosine phosphorylation of p85.

The Ras-related protein Rho might also be involved in mediating thrombin stimulation of PI3K. Activation of PI3K by GTP[γS] in platelet lysates was blocked by specific ADP-ribosylation of endogenous Rho by botulinium C3 exozyme. These effects were overcome by addition of exogenous recombinant Rho, but not by recombinant Rac [127]. Rho is also implicated in protein tyrosine phosphorylation and PI3K activation in Swiss 3T3 cells [128]. Rho-mediated stress-fibre formation in Swiss 3T3 cells is inhibited by genistein, suggesting that tyrosine kinase(s) could be downstream effectors of Rho [129].

p125^{FAK} (a **F**ocal **A**dhesion **K**inase) lies downstream of Rho activation [129]. Thrombin provokes FAK phosphorylation and activation in human platelets, in an integrin-dependent manner [99]. Previously identified as a cytosolic tyrosine kinase that associates with focal adhesion plaques upon stimulation by integrins and Src, this protein is implicated in the control of cytoskeletal reorganization processes. FAK is also implicated in PI3K activation [130]. It binds specifically to the p85 regulatory subunit of PI3K, and autophosphorylation of FAK increases the affinity of the association. Src and FAK appear to be functionally related, since FAK is a major tyrosine phosphorylated protein in Src-transformed cells [131], and autophosphorylation of FAK on Tyr^{397} (upon receptor binding) creates a high-affinity binding site for Src, so that FAK is recruited to an SH2 region of Src to initiate downstream signalling [132]. Ras binds specifically to p110, the catalytic subunit of PI3K, through its effector domain to stimulate PI3K activity [133,134]. The role of Ras, however, in the regulation of thrombin-stimulated PI3K remains uncertain.

A recent report has attempted to dissect the relative contributions of heterotrimeric G-proteins and monomeric G-proteins in regulating PI3Ks in thrombin-stimulated platelets [135]. A Rho-activatable p85/p110 PI3K, and a novel G$\beta\gamma$-sensitive p110γ PI3K that does not bind to p85 and thus functions independently of it, are activated in platelets in response to thrombin. The relationship, however, between this novel G$\beta\gamma$-sensitive p110γ PI3K and the G$\beta\gamma$-sensitive p85/p110 PI3K, also identified in platelets [136], remains to be elucidated.

(iii) Thrombin stimulation of Ras and Ras-related proteins

Thrombin stimulates Ras rapidly and transiently in a pertussis-toxin-sensitive manner in CCL39 cells, suggesting a role for the G$\beta\gamma$ subunits derived from G_i as upstream regulators of Ras [137]. Tyrosine kinases are also implicated in G_i activation of Ras, since thrombin stimulation of Ras is inhibited by the tyrosine kinase inhibitor genistein. Ras activation has been shown to be critical in thrombin-stimulated mitogenesis in 1321N1 astrocytoma cells [138], since microinjection of either a dominant negative Asn^{17} Ha-Ras mutant or an inhibitory antibody to Ras inhibits thrombin-stimulated DNA synthesis. It is noteworthy, however, that PtdIns(4,5)P_2 hydrolysis and intracellular Ca^{2+} mobilization were not dependent on endogenous Ras function.

Upon thrombin stimulation of human platelets the Ras-related protein Rap1b translocates to the cytoskeleton [139]. In the presence of extracellular Ca^{2+}, conditions that favour platelet aggregation, the thrombin-stimulated incorporation of Rap1b in the platelet cytoskeleton is biphasic and characterized by an initial rapid incorporation of approx. 20 % of the protein, followed by a sustained, but slower, second phase of incorporation lasting approx. 5 min [140]. In the absence of extracellular Ca^{2+}, conditions which inhibit aggregation, only the initial phase of Rap1b incorporation is observed [140]. The precise role of Rap1b in cytoskeletal dynamics is not known, although functionally Rap can compete with Ras for RasGAP and therefore might attenuate Ras signalling [141]. In this instance, however, p120RasGAP association with the cytoskeleton was not observed during thrombin stimulation. Rap2b also becomes associated with the platelet cytoskeleton upon thrombin stimulation [142]. Agonist-induced actin polymerization is required for the recruitment of Rap2b to the cytoskeleton, suggesting that Rap2b associates with newly synthesized actin filaments [143]. This association is dependent on glycoprotein IIb-IIIa, since inhibitory monoclonal antibodies directed towards this receptor inhibit Rap2b recruitment to the cytoskeleton [143].

Rho is important in regulating integrin-dependent formation of stress fibres at focal adhesion complexes [112] and has been implicated in thrombin-stimulated neurite retraction in N1E-115 and NG108-15 neuronal cells [144] and in thrombin-induced

platelet aggregation mediated by glycoprotein IIb-IIIa [145]. The molecules linking thrombin-receptor activation to Rho activation are not yet known, although it is apparent that Rho can regulate multiple effectors with tyrosine kinases, PI3K, PLD and PtdIns4*P* 5-kinase all lying downstream of Rho activation. The Rho-related protein Rac, integral in lamellipodia formation and membrane ruffling [146], has also been implicated as a downstream effector of PI3K [147,148], although it is not known whether the thrombin receptor can thus couple to Rac activation.

(iv) Thrombin stimulation of MAP kinases

MAP kinases, which possess serine, threonine and tyrosine kinase activity, are important in mediating responses to both tyrosine kinase receptors and G-protein-coupled receptors. They are activated by kinase cascades that are initiated following Raf activation by Ras (reviewed in [149]). Raf is a specific MAP kinase kinase kinase (MAPkkk), which phosphorylates (on serine and threonine residues) and activates MAP kinase kinase (MAPkk) [150], which in turn phosphorylates and activates $p42^{mapk}$ and $p44^{mapk}$ [151].

Thrombin has been shown to stimulate both $p42^{mapk}$ and $p44^{mapk}$ activity, although it differentially stimulates $p44^{mapk}$ in G_0-phase-arrested CCL39 cells [49]. $p44^{mapk}$ stimulation is defined by an initial transient activation that is maximal after 5 min, followed by a second period of activation that lasts up to 4 h. The prolonged phase of MAP kinase activation correlates well with thrombin-stimulated DNA synthesis. Interestingly TRAP transiently stimulates $p44^{mapk}$, but fails to induce the prolonged phase of activation and fails to stimulate DNA synthesis [see section 3(iii)]. Conversely, in human platelets, thrombin selectively stimulates $p42^{mapk}$, but not $p44^{mapk}$ [152], an activation that parallels $p90^{rsk}$ activation. These responses were shown to be independent of integrin-mediated platelet aggregation [152]. This suggests that $p42^{mapk}$ and $p90^{rsk}$ might be important in regulating cytoskeletal changes that accompany platelet activation, whereas $p44^{mapk}$ might be more directly involved in mitogenesis.

(v) Thrombin stimulation of phosphatidylcholine (PtdCho) breakdown

Thrombin stimulation of human platelets causes activation of PLA_2. This enzyme is responsible for the liberation of arachidonic acid, predominantly from PtdCho, which is required for the synthesis of thromboxane A_2 and leukotrienes. PLA_2 is found in two forms: $sPLA_2$, a secreted protein that is activated by millimolar Ca^{2+}, and $cPLA_2$, a cytosolic species that is activated by micromolar Ca^{2+} and is characterized by a Ca^{2+}-dependent phospholipid-binding motif in its N-terminal domain. An $sPLA_2$ that is secreted within minutes of thrombin stimulation of human platelets has been cloned [153], although since thromboxane A_2 production in human platelets is maximal at low external Ca^{2+}, this form is perhaps not critical in early arachidonic acid generation. An 85 kDa $cPLA_2$ species present in human platelets is also stimulated by thrombin. $p42^{mapk}$ has been shown to stimulate $cPLA_2$ activity by directly phosphorylating $cPLA_2$ on serine residues. Ca^{2+} is also required for activity, which serves to localize $cPLA_2$ to membranes [154,155]. Activation of $cPLA_2$ positively correlates with thrombin-stimulated phosphorylation of $cPLA_2$ [156]. G-proteins are also implicated in $cPLA_2$ activation. Thrombin-stimulated $cPLA_2$ in CHO cells has been shown to be dependent on functional $G\alpha_{i2}$ proteins, since a $G\alpha_{i2}$ mutant, G203T, substantially inhibits $cPLA_2$ activation [157].

PLD stimulates the generation of phosphatidate primarily from PtdCho and exists as both membrane-associated and cytosolic species [158]. Two distinct membrane-associated PLDs have been identified [159]. One form is oleate-dependent [159,160], whereas the other is stimulated by the low-molecular-mass G-proteins ARF and Rho [159,161] and requires PtdIns(4,5)P_2 as an obligatory cofactor [159,162]; this serves to accelerate GDP dissociation from ARF [163]. Phosphatidate is involved in thrombin-stimulated stress-fibre formation in IIC9 fibroblasts [164], characterized by an increase in actin polymerization. Tyrosine kinases are implicated in thrombin stimulation of PLD in platelets, since the tyrosine kinase inhibitors genistein and the tyrphostins A25 and A47 inhibit the activity of the thrombin-stimulated enzyme. Moreover, in saponin-permeabilized platelets, tyrosine kinase inhibitors markedly reduce GTP[γS]-stimulated PLD activity, suggesting that tyrosine kinase activation of PLD lies downstream of a regulatory G-protein [165].

Thrombin also stimulates DAG accumulation in IIC9 fibroblasts, characterized by the transient translocation of PKCα and the biphasic translocation of PKCϵ to a membrane fraction. DAG from PtdIns(4,5)P_2 hydrolysis accounts for the initial phase of PKCα and PKCϵ activation, with DAG derived from PtdCho responsible for prolonged phase of PKCϵ activation [166]. The differences in selective PKC isoenzyme activation do not reside in DAG species differences [166], but can be explained by the requirement for Ca^{2+} for PKCα activation but not for PKCϵ activation [166]. The thrombin-stimulated accumulation of nuclear DAGs in IIC9 fibroblasts correlates positively with the translocation of PKCα to the nucleus [167].

(vi) Thrombin inhibition of adenylate cyclase

Agonists such as prostaglandins, which couple to adenylate cyclase to elevate intracellular cyclic AMP levels, inhibit platelet activation [168]. Thrombin inhibits agonist-stimulated adenylate cyclase activity in both membrane preparations and intact human platelets, thus reducing intracellular cyclic AMP levels [85]. Moreover, thrombin markedly reduces forskolin-stimulated adenylate cyclase activity in CCL39 fibroblasts, whilst pertussis toxin pretreatment abolishes thrombin-mediated inhibition, implicating G_i in the control of adenylate cyclase [169]. $G\alpha_z$, found predominantly in neuronal tissue and platelets, shows 65% sequence similarity at the amino acid level with $G\alpha_i$ members, but is insensitive to pertussis-toxin-catalysed ADP-ribosylation [170,171]. Thrombin and phorbol 12-myristate 13-acetate stimulate $G\alpha_z$ phosphorylation in platelets, and purified recombinant $G\alpha_z$ is a substrate for PKC *in vitro*, whereas $G\alpha_i$ members are not [172]. $G\alpha_z$ has recently been shown to inhibit adenylate cyclase activity [173].

Thrombin-mediated inhibition of adenylate cyclase effectively reduces intracellular cyclic AMP levels, presumably reducing cyclic AMP-dependent protein kinase A activity. Elevation of intracellular cyclic AMP also functions to inhibit Raf activity [174] such that thrombin might mediate Raf activation not only through Ras, but also by relieving cyclic AMP-mediated inhibition. Thrombin can also potentiate prostaglandin-stimulated adenylate cyclase activity in HEL cells by a mechanism necessitating PKC activation [175].

5. CELLULAR RESPONSES TO THROMBIN

As we have mentioned above, thrombin has the remarkable ability to interact with a large variety of cell types. Furthermore, depending on the cellular target, it can rapidly elicit a range of physiological responses in addition to the events leading to cell

division [176–183]. Many of these actions of thrombin are precisely those that would be required during inflammation, tissue remodelling and eventual wound repair. For example, thrombin is involved in the inflammatory response by causing chemotaxis and adhesion of inflammatory cells [177,184], it invokes contraction and tissue remodelling by inducing morphological changes in endothelial cells [185] and fibroblasts [186], and it contributes to wound healing by stimulating mitogenesis, either directly [187] and/or via its ability to induce the secretion of other growth factors [183]. Thus thrombin plays a central role in a second 'cascade' of events following injury, a cascade of post-clotting cellular effects whereby the enzyme acts, in concert with other molecules, as a hormone and growth factor. Limitations of space preclude a detailed discussion of all of the cell types which respond to thrombin, and so we have concentrated in this section on a consideration of the role of thrombin in the regulation of growth of cells of neuronal origin, as this is an area which seems to have received rather less attention than it merits.

(i) Thrombin in the nervous system

Although the blood/brain barrier segregates macromolecules between the brain and the vascular system, evidence has accumulated for an important function for thrombin and other serine proteinases in the brain (reviewed in [188]). Most graphic, perhaps, is the effect of thrombin on the morphology of various cultured cell types derived from the nervous system. The response is typified by a rapid (visible within minutes) retraction of neurites or processes and can be induced by picomolar concentrations of thrombin or nanomolar concentrations of its precursor prothrombin. Furthermore, it is specific to thrombin, since other serine proteinases are either ineffective or result in general proteolysis and cell detachment. Interestingly, another serum component, namely lysophosphatidic acid (LPA), which also couples to a G-protein-coupled receptor, triggers rapid neuronal shape changes that are indistinguishable from those induced by thrombin [189]. To date, neurite retraction has been demonstrated in a variety of neuronal cells, including mouse neuroblastoma cells [10], the SK-N-SH human neuroblastoma cell line, human transformed fetal retinoblasts (HER 10), primary cultures of human fetal cerebellar, hippocampal and midbrain neurons [190], primary cultures of neonatal rat brain (Figure 6), hippocampal pyramidal cell cultures [191] and primary cultures of fetal dopaminergic neurons [192]. A comparable response has also been demonstrated in cells of glial lineage. Astrocytes and glioma cells rapidly lose their stellate morphology and acquire a flat epithelial shape upon addition of picomolar concentrations of thrombin [193–195]. Astrocytes participate in several processes crucial to brain function (reviewed in [196]). They may provide

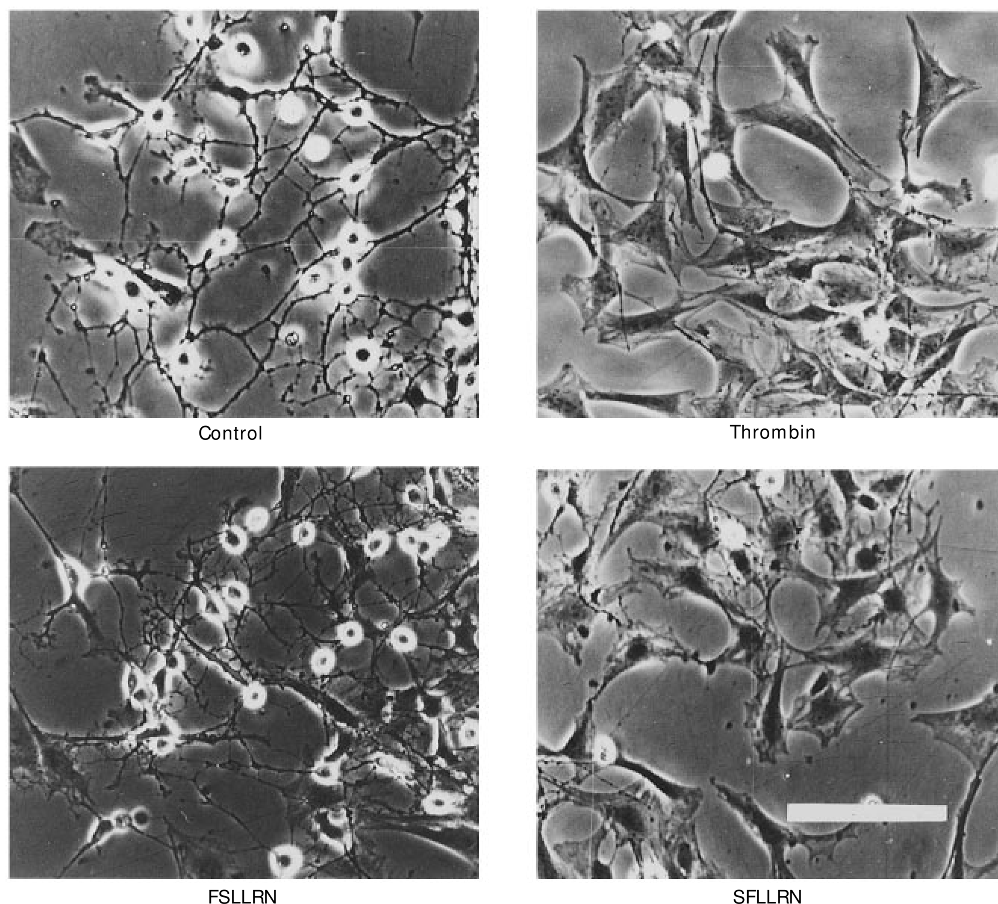

Figure 6 Thrombin- and TRAP-induced neurite retraction in primary cultures of rat neonatal brain

Cells were differentiated by treatment with 30 μM forskolin in the absence of serum for 4 h. Thrombin (28 pM), TRAP-SFLLRNP (10 μM) or control peptide FSLLRNP (10 μM) were added to cultures and observed 2 h later (as shown). The bar represents 50 μm (P. W. Grabham and D. D. Cunningham, unpublished work).

trophic support for neuronal growth and differentiation *in vivo* by secreting neurotrophins such as nerve growth factor (NGF) [197] and FGF [198], and it has been suggested that astrocytes regulate synaptic remodelling of neurosecretory neurons [199,200]. Moreover, astrocytes are believed to contribute to the blood/brain barrier [201]. In addition to the reversal of stellation, thrombin is known to induce other responses in astrocytes. For example, it causes secretion of the potent vasoconstrictor endothelin-1 [202], it has recently been demonstrated to enhance secretion and synthesis of NGF [203] and it can act as a growth factor on astrocytes [193–204]. Thus many of the post-clotting cellular effects of thrombin seen in the vasculature may be reproduced within the brain. Furthermore, morphological changes in neurons and astrocytes and mitosis in astrocytes have been shown to be mediated by the tethered ligand receptor [48,205–207], indicating that these responses in brain cells are not due to general proteolytic effects, but involve the receptor-signalling mechanisms outlined above.

The precise second-messenger pathway(s) by which activation of the cloned receptor leads to morphological change in neuronal cells remains to be elucidated. Pharmacolgical studies have shown the involvement of serine/threonine kinase(s), whilst experiments using tyrosine kinases and phosphatase inhibitors indicate that a receptor-mediated pathway associated with these enzymes might transmit the response [48]. The reversal of astrocyte stellation, however, is not sensitive to the tyrosine kinase inhibitor herbimycin A, suggesting that certain tyrosine kinases are probably not involved in this process [208]. Additionally, in adenovirus-transformed HER10 cells, thrombin-induced neurite retraction is accompanied by PIC and PI3K activation [59], suggesting that at least some of the responses to thrombin in neuronal cells are the same as those seen in platelets and fibroblasts. In any event it is likely that regulation of the actin-based cytoskeleton is crucial to thrombin and LPA-induced morphological changes [189], and recent studies on N1E-115 and NG108-15 neuronal cells indicated that Rho mediates this process [144] [section 4 (iii)].

Evidence for the importance of thrombin effects on neuronal cells *in vivo* comes from studies of proteinase nexin-1 (PN-1), also known as glia-derived nexin (GDN) (reviewed in [208]), a serine-proteinase inhibitor that has highest affinity for thrombin and is found primarily in the brain [209,210]. PN-1/GDN inactivates thrombin by forming a tight complex which subsequently binds back to the cells, where it is rapidly internalized and degraded [211]. Thus PN-1/GDN counteracts the cellular effects of thrombin, inhibiting mitogenic activity and inducing neurite extension in neurons and stellation in astrocytes. Indeed, the neurite-promoting action of PN-1/GDN [212,213], which corresponds directly with its inhibition of the enzyme activity of thrombin [214], led to the hypothesis that neuronal morphology is governed by a delicate interplay of proteinase and inhibitor [215].

(ii) Injury-related actions of thrombin in the brain

A compromise of the blood/brain barrier during disease or injury would, in theory, expose cells in the brain to relatively large amounts of thrombin and other plasma proteins, with obvious deleterious effects [216–218]. Recent studies have demonstrated expression of thrombin-receptor mRNA in rat brain, strongly suggesting that the extensive morphological alterations caused by thrombin on neurons and astrocytes in culture also occur *in vivo*. Such activity could disrupt critical interactions between neurons (synapses) and between astrocytes and either neurons or capillaries. Furthermore, the mitogenic activity of thrombin on astrocytes in culture might, *in vivo*, contribute to the gliosis commonly observed after central-nervous-system (CNS) injury. Indeed, it has recently been shown that infusion of thrombin into rat brain results in histological changes that resemble the inflammation, scar formation and reactive gliosis seen in the CNS following injury [219].

In the brain PN-1/GDN is secreted mainly by astrocytes [220] and is found at sites that indicate a role in maintaining the integrity of the blood/brain barrier under normal conditions by excluding thrombin from the brain. For example, it is concentrated around cerebral blood vessels and associated with the endfeet of astroglia that form tight junctions with endothelial cells around capillaries [221]. Since the concentration of prothrombin in plasma is in the micromolar range and the concentration of PN-1/GDN in the brain is relatively low [221], extravasation of plasma through injury or disease would be expected to result in an imbalance in favour of thrombin and lead to the deleterious effects described above. There is, however, evidence that injury and disease-related events trigger PN-1/GDN production to levels which ultimately negate the effects of thrombin. First, a marked increase in PN-1/GDN mRNA has been found in biopsies of human glioblastoma and astrocytoma compared with normal brain [222]. Secondly, it has been shown that forebrain ischaemia in gerbils leads to alterations in the blood/brain barrier, selective degeneration of hippocampal CA1 pyramidal neurons and increased immunoreactivity to PN-1/GDN [223]. Thirdly, astroglial cells in the substantia nigra synthesize PN-1/GDN *de novo* following cytotoxic insult [224]. Finally, glial cells in culture have been shown to upregulate their secretion of PN-1/GDN in response to injury-related factors such as interleukin-1 and tumour necrosis factor-β [225].

While excess, unregulated concentrations of thrombin are undoubtedly damaging, the half-maximal concentration of thrombin required for proliferation in astrocytes (500 pM) is 100-fold higher than that required for morphological changes in astrocytes (2 pM) and neurons (2–10 pM) [10,190,193]. These observations suggest that thrombin might have a differential effect *in vivo*, depending on its concentration. It has therefore been postulated that a low concentration of thrombin may, in fact, contribute to the repair process by increasing plasticity and aiding the reconstruction of damaged synapses and other intercellular connections. Furthermore, PN-1/GDN might participate in this process by regulating the levels of active thrombin in the immediate environment of both neurons and astrocytes [208].

(iii) A function for thrombin in the normal brain

A major unanswered question concerning the action of thrombin in the brain is whether it has a function under normal physiological conditions. Although there is convincing evidence for a type of wound response and ultimately reactive gliosis, as described in the previous section, the evidence for a function under normal circumstances is scant and conjectural. Perhaps most persuasive are observations that prothrombin mRNA is present in brain and in cell lines of neuronal and glial origin [226], indicating that, in addition to injury-related extravasated thrombin, there is a potential endogenous source in the brain. It should, however, be noted that this has not yet been demonstrated directly.

The distribution of prothrombin mRNA in brain provides clues to the biology of a possible normal function of thrombin in the CNS. Expression has been observed in a number of embryonic and early postnatal rat brain structures, including the olfactory bulb, cortex, hippocampus, striatum, colliculi, thalamus and substantia nigra [217,226]. A comparison of the pattern of

prothrombin and thrombin-receptor mRNA has revealed a distribution that is distinct, but overlapping, in select brain regions. For example, in cerebellum, thrombin-receptor mRNA is localized to the Purkinje-cell layer, whereas prothrombin mRNA is localized to both Purkinje- and granule-cell layers [217]. In most cases, thrombin mRNA and receptor mRNA expression are in close proximity, indicating that locally synthesized thrombin could act in an autocrine or paracrine manner to regulate specific processes. Further clues to a normal function for thrombin in the brain can, once again, be found in studies on PN-1/GDN. In addition to the protective role it might play in pathological situations, it is likely that the inhibitor also modulates the activity of locally synthesized thrombin during normal processes such as synaptic remodelling. Expression of PN-1/GDN mRNA and protein have distinct spatial and temporal patterns that support this theory [210,227,228]. For example, PN-1/GDN levels are high in the olfactory bulb, where glial cells and neurons proliferate postnatally and there is continual ingrowth of sensory axons and formation of new synapses into adulthood [229]. Furthermore, levels of prothrombin mRNA and thrombin-receptor mRNA are also high in this region [217].

Perhaps the most convincing role for thrombin is during development. This is a period when cell division among both neuronal and glial cells is maximal, remodelling and migration occur widely, and the blood/brain barrier is not yet intact. Indeed, formation of the blood/brain barrier in the rat occurs gradually during embryogenesis, and complete closure to macromolecules is not achieved until the third postnatal week [230]. Studies on the expression of rat thrombin-receptor mRNA [217,218], prothrombin mRNA [240] and PN-1/GDN [209,228] all indicate a correlation with age, where levels are highest during ontogeny and thence decrease to a basal level in adult life.

The precise nature of a physiological action for thrombin at the cellular level remains unknown. Current knowledge, however, suggests that thrombin-induced morphological changes represent a response that occurs under normal conditions. Although PN-1 is abundant around vessels, it is present to a lesser extent in the brain [221], indicating that very low concentrations of thrombin are normally present (this is probably why prothrombin has not, to date, been detected either *in vivo* or in neuronal cell lines in culture). Tissue-culture experiments show that much lower (picomolar) concentrations of thrombin are required for neurite retraction and the reversal of stellation than for mitogenesis ([10,59,190,193]; D. P. Brant and R. J. A. Grand, unpublished work). Moreover, the notion that neurite retraction is separate from other responses is supported by the observation that stimulation of distinct second-messenger pathways occurs in these responses [48,59,205,207]. Thus it is possible that small quantities of prothrombin are produced locally and converted into thrombin, which then acts locally to modify the morphology of neurons and astrocytes. Such activity, in exquisite co-ordination with PN-1/GDN, might contribute to cytoarchitectural remodelling and cellular migration in both the developing and adult brain.

(iv) Biological activity of prothrombin in the brain

Prothrombin appears to have no inherent proteolytic activity and has generally been considered to be biologically inactive prior to cleavage. However, it has been known for some time that it can induce neurite retraction [190,231] and the reversal of astrocyte stellation (P. W. Grabham and D. Cunningham, unpublished work) in the absence of other serum factors. This raises the possibility that the proenzyme can act directly on cells in the brain to elicit a biological effect. However, the weight of evidence so far accumulated suggests that prothrombin must be converted into thrombin for it to evoke a response. Thus GDN and hirudin inhibit prothrombin-mediated neurite retraction even though they are incapable of binding to the proenzyme and only interact with thrombin [59,232]. Additionally, prothrombin elicits many of the intracellular responses seen with thrombin. For example, stimulation of cells with either protein rapidly activates a similar set of protein tyrosine kinases and PI3K. However, certain differences in intracellular responses to the two proteins have been noted. In neurite-retraction assays, appreciably higher concentrations of prothrombin than thrombin are required to produce a similar response [59,190]. Stimulation of cells with prothrombin does not cause detectable activation of PIC nor rapid synchronized Ca^{2+} mobilization [59]. On the basis of these data it could be suggested that cellular metabolic responses to prothrombin duplicate those elicited by very low thrombin concentrations. This is consistent with small amounts of thrombin being formed by cleavage of prothrombin at the cell surface [59,190] and, indeed, recent evidence has indicated the Ca^{2+}-dependent conversion of proenzyme into enzyme on the surface of human neuronal cells [59] and feline kidney fibroblasts [233]. It is not clear whether a specific enzyme, analogous to Factor Xa, is present on the surface of responsive cells or whether a more general proteinase is involved in activation.

6. FUTURE PROSPECTS

One of the most engaging features of thrombin research is the realization that an enzyme which is usually encountered as simply a member of the coagulation cascade can markedly influence the behaviour of a wide range of mammalian cell types which ostensibly have little to do with the clotting process. The observation that cells as diverse as platelets and astrocytes will respond to thrombin has confirmed that the protein has a hormone-like activity, allowing it to regulate the growth patterns in an array of targets. The elegant characterization of the thrombin receptor by groups led by S. R. Coughlin, J. Pouyssegur and L. F. Brass over the past 5 years has gone a long way towards explaining how the proteinase is able to produce this range of cellular responses. Additionally this work has provided a lucid account of the events which occur at the cell surface as a result of thrombin proteinase activity.

It now seems that the major areas of uncertainty in our understanding of thrombin signalling lie downstream of receptor activation. In particular, in the immediate future it will be important to understand whether separate pathways are involved in mitogenesis (in which transcriptional activation occurs) and those responses which only result in modification of the cytoskeletal architecture (i.e. cellular shape changes through reorganization of actin filaments). Indeed, preliminary evidence has already been presented suggesting that $p44^{mapk}$ and $p42^{mapk}$ may have distinct roles in separate thrombin-stimulated signalling pathways, only some of which are involved in proliferation [49,153]. Additionally, we need to question the significance of the roles played by Ras-related proteins in thrombin responses. It may be that some components (e.g. Ras itself) are involved in transduction of signals to the nucleus, whilst others may be central to 'shape changes', for example the role of Rho in neurite retraction [144].

A further point of considerable interest is the relative importance of G-protein-coupled and tyrosine kinase-mediated responses triggered by the thrombin receptor. Do these biochemical events form part of the same signalling pathway or are

they quite distinct? In the event of the latter possibility does this mean that proteinase-activated receptors can (uniquely?) activate tyrosine kinases without recruiting G-proteins?

Whilst most interest has obviously focussed on the action of thrombin on platelets, in this review we have tried to emphasize the ubiquitous nature of the thrombin response. Although there have been demonstrations of the regulation by thrombin of growth and differentiation in cells of neuronal origin in tissue-culture systems, these experiments have not yet been extended to show unequivocally that the proteinase can determine cell morphology *in vivo*. This remains a priority. In this context it is a matter of some urgency to understand how prothrombin might be processed on the surface of cells, since it is likely that, under certain circumstances *in vivo* (for example in the brain), this might serve as the only source of thrombin.

A number of cell-surface proteinases have been reported, however (see, for example, [234,235]). Although their biochemical functions are not clear, they play roles in cell growth, cell invasion and tissue rearrangement. Specific protein substrates for these proteinases remain unknown, but it might be supposed that one or more might be able to convert prothrombin into thrombin. Hepsin [237,238], for example, has been shown to be essential for cell growth of human hepatoma cells [238], and it is possible that it is required for the generation of thrombin from the added bovine serum in culture.

With the recent cloning of a trypsin-sensitive receptor homologous with the thrombin receptor [239], it now seems possible that a whole family of proteinase-activated cell-surface receptors may be present in mammalian cells [240]. An understanding of their mode of action and the relationship between them and the thrombin receptor opens up a large new area of exciting research possibilities.

Note added in proof (received 18 October 1995)

Hartwig et al. [241] have recently proposed a role for the D3 and D4 polyphosphoinositides in mediating thrombin-stimulated actin polymerisation in human platelets. Specifically, PtdIns4P, PtdIns(4,5)P_2, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 stimulate the 'uncapping' of barbed (fast-growing) actin filaments, allowing for the further extension of actin polymers. The low-molecular-mass G-protein Rac plays a crucial role in this process by stimulating PtdIns(4,5)P_2 synthesis, thus confirming its role in thrombin-mediated morphological changes.

We thank Professor Dennis Cunningham and Dr. Patrick Vaughan (University of California) for their encouragement and help. We are also most grateful to Dr. Julian Parkhill and Dr. David Molloy, Institute for Cancer Studies, for assistance with the computer graphics, Dr. Matt Hodgkin for critical reading of parts of the manuscript before its submission, and Nicola Waldron and Deborah Williams for excellent secretarial assistance. We thank the Cancer Research Campaign for financial support (to R.J.A. G. and A.S.T.).

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