Thrombin regulates S-phase re-entry by cultured newt myotubes

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Background: Adult urodele amphibians such as the newt have remarkable regenerative ability, and a critical aspect of this is the ability of differentiated cells to re-enter the cell cycle and lose their differentiated characteristics. Unlike mammalian myotubes, cultured newt myotubes are able to enter and traverse S phase, following serum stimulation, by a pathway leading to phosphorylation of the retinoblastoma protein. The extracellular regulation of this pathway is unknown.

Results: Like their mammalian counterparts, newt myotubes were refractory to mitogenic growth factors such as the platelet-derived growth factor (PDGF), which act on their mononucleate precursor cells. Cultured newt myotubes were activated to enter S phase by purified thrombin in the presence of sub-threshold amounts of serum. The activation proceeded by an indirect mechanism in which thrombin cleaved components in serum to generate a ligand that acted directly on the myotubes. The ligand was identified as a second activity present in preparations of crude thrombin and that was active after removal of all thrombin activity. It induced newt myotubes to enter S phase in serum-free medium, and it acted on myotubes but not on the mononucleate precursor cells. Cultured mouse myotubes were refractory to this indirect mechanism of S-phase re-entry.

Conclusions: These results provide a link between reversal of differentiation and the acute events of wound healing. The urodele myotube responds to a ligand generated downstream of thrombin activation and re-enters the cell cycle. Although this ligand can be generated in mammalian sera, the mammalian myotube is unresponsive. These results provide a model at the cellular level for the difference in regenerative ability between urodeles and mammals.

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Background

Adult urodele amphibians such as the newt and axolotl are capable of regenerating significant parts of the body plan such as the limb, tail and jaw, as well as tissues such as the lens, retina, iris and substantial sections of the heart [1,2]. A key characteristic in these various contexts is the ability of differentiated cells to re-enter the cell cycle in response to local injury or tissue loss, and to lose their differentiated characteristics in a reversible fashion. For example, removal of the lens elicits these changes in the pigmented epithelial cells of the dorsal iris [3,4], whereas cardiac lesions provoke extensive division of atrial and ventricular cardiomyocytes [5,6]. Regeneration of the limb and jaw involves the local response of mesenchymal cells to form a growth zone or blastema underlying the wound epidermis [7,8]. Although it is not possible to exclude some contribution from reserve cells, the widespread plasticity of differentiated cells appears to be an important mechanism underlying the difference in regenerative ability between urodeles and other vertebrates such as mammals.

It is unclear as to what extent differentiated urodele cells might be intrinsically different from their mammalian counterparts in their regulation or responsiveness, and to what extent they might be exposed to distinct extracellular signals on initiating regeneration. We have addressed these questions in the context of newt skeletal myotubes, which form readily in cultures of mononucleate limb A1 cells when the serum concentration of the medium is lowered [9–11]. When the multinucleate myotubes are selectively labelled by microinjection of a lineage tracer and introduced into a blastema, they give rise to labelled mononucleate cells which divide and contribute to the regenerate [10]. These observations have recently been confirmed using myotubes labelled either with an integrated retrovirus or with a cell-tracker dye. Furthermore, the myotube nuclei are found to re-enter S phase after implantation (A. Kumar, C. Velloso, Y. Imokawa and J.P.B., unpublished observations). The behaviour of the implanted myotubes is reminiscent of earlier observations of endogenous muscle fibres during limb regeneration [12,13]. These results establish that A1 myotubes are appropriate target cells for studying the mechanisms underlying the reversibility of differentiation.

In culture, these newt myotubes have a distinctive feature that distinguishes them from their avian and mammalian counterparts. A characteristic of vertebrate myogenesis is

the entry into a stable post-mitotic arrest after fusion [14,15]. This leaves the multinucleate myotubes completely unresponsive either to serum or to protein growth factors which act to stimulate S-phase entry of mononucleate myoblasts [16,17]. Newt myotubes maintained in low serum media also remain stably arrested indefinitely. When cultured newt myotubes are exposed to elevated concentrations of serum, however, they have been observed to traverse S phase and arrest in G2 [11]. What is the basis for this difference between newt and mammalian myotubes? The activity of the retinoblastoma protein (Rb) is critical for stable withdrawal of multinucleate muscle cells from the cell cycle. In normal mouse myotubes, the pathway leading to cell-cycle re-entry through hyperphosphorylation of Rb is blocked [18], whereas in mouse myotubes lacking both copies of the *Rb* gene, serum does stimulate re-entry [19,20]. In newt A1 myotubes, the appearance of the hyperphosphorylated, inactive form of Rb underlies their serum response, as expression of p16, an inhibitor of cyclin-dependent kinases (Cdks) 4 and 6, effectively blocks S-phase re-entry [11]. These experiments provide clear evidence for an intrinsic difference between urodele and mammalian myotubes, while raising the question as to what activity in serum might be responsible for activating the Rb pathway in newt cells.

The stimulation of S-phase entry in a cycling or quiescent cell such as the fibroblast is a familiar aspect of the response to serum, but the myotube is a differentiated cell, a circumstance which makes this example of particular interest. Cells generally encounter serum, the soluble fraction of clotted blood, in the context of wounding followed by thrombin activation and haemostasis. The recent analysis of the fibroblast response to serum, by hybridisation of RNA to DNA microarrays, has revealed that many of the genes that are activated are familiar players involved in wound healing [21]. The response of urodele myotubes is thus of additional interest because, as discussed later, wounding is thought to be an important trigger for the responses leading to regeneration [22]. In this paper, we have characterised the serum activity that elicits newt myotube cell-cycle re-entry, and report that the serum protease thrombin acts as an indirect regulator by generating a ligand that is active on the myotube but not on the mononucleate precursor cell. The results provide an intriguing model for analysis at the level of cell biology of the difference in regenerative ability between urodeles and mammals.

Results

Responsive and refractory properties of newt and mouse myotubes

All sources of animal sera that we tested were capable of inducing cultured newt A1 myotubes expressing myosin heavy chain marker to re-enter S phase; yet when, for example, foetal bovine serum was tested in parallel on mouse C2C12 myotubes, it was unable to induce S-phase re-entry in these cells (Table 1). In contrast, serum was a powerful mitogenic stimulus for the mononucleate muscle precursors of both newt and mouse (Table 1). The ability of serum to induce S phase in cycling and quiescent cells usually reflects the activity of mitogenic growth factors such as the platelet-derived growth factor (PDGF), but, in agreement with previous results [11], a variety of mammalian growth factors including PDGF were found to be inactive on A1 myotubes while stimulating the mononucleate newt cells (see Table 1). This indicates first that the post-mitotic arrest in newt myotubes — defined by the loss of responsiveness to mitogenic factors after fusion — is quite comparable to that in mammalian myotubes such as C2C12 cells, and second, that the ability of serum to countermand the post-mitotic arrest and provoke S-phase re-entry in newt myotubes is not due to the growth factors tested, whether singly or in a variety of combinations (see Table 1).

Thrombin promotes re-entry into S phase

In view of the distinctive nature of the serum activity and its potential importance for dedifferentiation and regeneration, we assayed not only a variety of purified factors but also certain crude fractions derived from plasma or serum.

Table 1

Sensitivity of mouse C2C12 myotubes and newt A1 myotubes to various growth factors.

Cell type	PDGF*	Serum†
Mouse C2C12 cells		
Mononucleate	+	+
Myotube	–	–
Newt A1 cells		
Mononucleate	+	+
Myotube	–	+

Myotubes were assayed for bromodeoxyuridine (BrdU) incorporation in response to growth factors or serum. *PDGF was found to be active on newt A1 mononucleate cells but inactive on newt myotubes. Other growth factors with the same properties include: basic fibroblast growth factor (bFGF, 10 ng/ml); keratinocyte growth factor (10–50 ng/ml); epidermal growth factor (EGF, 50 ng/ml); and insulin-like growth factor-1 (IGF-1; 100 ng/ml). The following growth factors and other substances were assayed solely on myotubes and found to be inactive: tumour necrosis factor α (TNF-α, 2000 U/ml); activin A (10–20 ng/ml); macrophage stimulating protein (3–30 nM); hepatocyte growth factor (2–10 U/ml); murine colony stimulating factor (mCSF, 5 U/ml); glial growth factor (10–20 ng/ml); retinoic acid (0.1–1.0 nM); lysophosphatidic acid (2–20 ng/ml); sonic hedgehog (0.4–40 μg/ml); thrombin-receptor agonist peptide (1–100 nM); and platelet lysate. Transforming growth factor β (TGF-β, 200 U/ml) was inactive on myotubes and induced cell-cycle arrest in mononucleate cells. †Serum sources tested (in addition to foetal bovine serum) and found to be active include adult bovine, sheep, porcine and chicken sera. We were unable to obtain sufficient quantities of urodele sera for assay. In assays on mononucleate A1 cells, a plus sign represents a greater than fivefold increase in the number of BrdU-positive cells compared with media containing 1% serum. In assays on myotubes, a minus sign means less than 5% BrdU-positive cells in an assay in which serum induced 30% BrdU-positive cells.

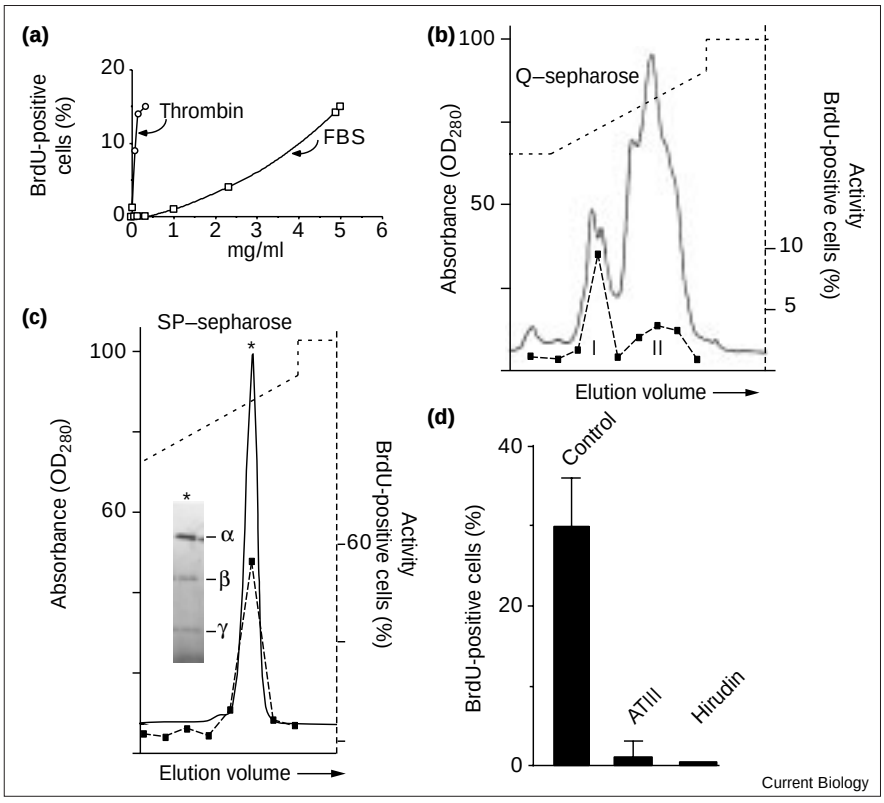
For example, platelet lysate, a potent source of activities on cycling and quiescent cells, was tested but found to have no detectable 're-entry activity' on A1 myotubes. One plasma derivative, however, contained significant S-phase re-entry activity. Commercial preparations of crude thrombin, prepared from bovine plasma, were found to be active on newt myotubes in the concentration range 10–100 μg/ml, whereas foetal bovine serum was active at 1–5 mg/ml (Figure 1a). When crude thrombin was fractionated by adsorption to and elution from an anion exchange resin, Q–sepharose, two peaks of activity were obtained (Figure 1b). Peak I, which eluted at 200 mM NaCl, was further chromatographed on a cation exchange resin, SP–sepharose, and eluted at 500 mM NaCl as a single peak of activity co-incident with the major peak of protein (Figure 1c). After SDS gel electrophoresis and western blotting this was found to contain highly purified thrombin (Figure 1).

The dependence of S-phase re-entry on thrombin activity was established using a panel of protease inhibitors. Proteolytic activity was removed by prior treatment of the SP–sepharose fraction with either of two protein inhibitors — anti-thrombin III (ATIII), which acts at the active site of several serine proteases [23], and hirudin, which acts at the fibrinogen-binding exosite of thrombin [24] (Figure 1d). A small-molecule inhibitor, PPACK, which alkylates the histidine residue of the catalytic centre [25], also completely removed proteolytic activity at a concentration of 10 μM (data not shown). All of these inhibitor-treated samples had no significant residual activity on the newt myotubes.

Thrombin-mediated re-entry requires serum

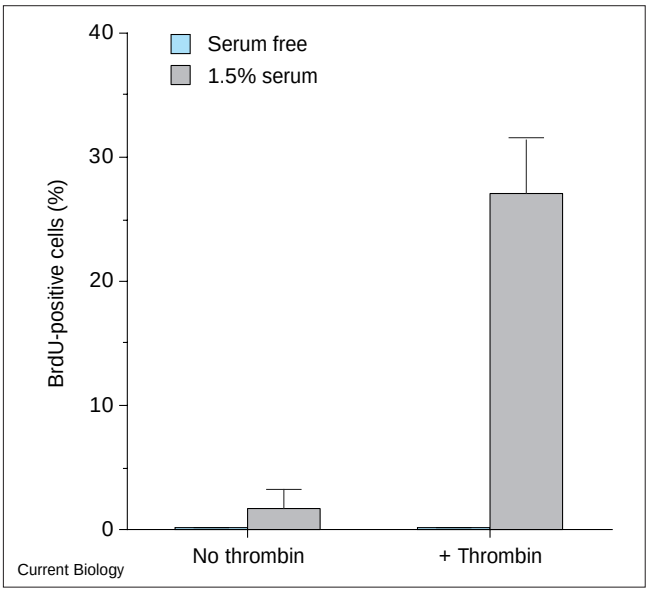
An important aspect of the action of thrombin on newt myotubes is illustrated in Figure 2. When myotubes were cultured under conditions in which serum was replaced with 1% crystalline bovine serum albumin (BSA), the myotubes were refractory to thrombin stimulation, whereas thrombin strongly stimulated re-entry in the presence of sub-threshold concentrations of serum. In control experiments, myotubes cultured under serum-free conditions were found to be fully responsive to serum stimulation, and, as shown later, to partially purified protein fractions. Thrombin could either be acting directly on the

Figure 1



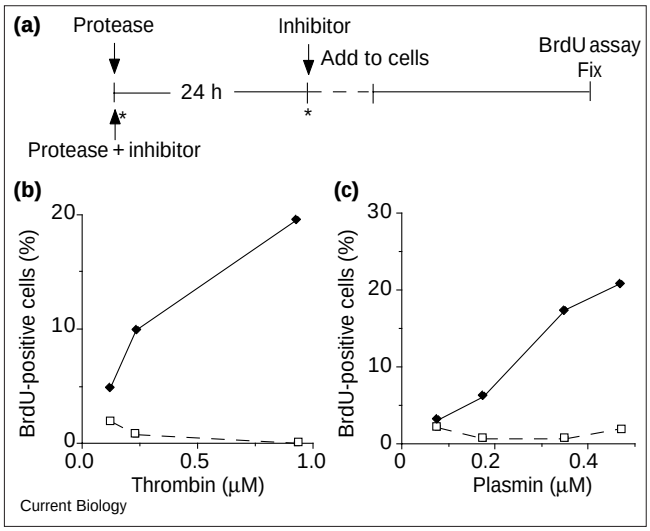
Identification of bovine thrombin as a protein that elicits newt myotube cell cycle re-entry. (a) Myotube S-phase re-entry activity of a crude bovine thrombin preparation compared with foetal bovine serum (FBS). Activity was assayed as the percentage of myotubes that were BrdU-positive. (b) The re-entry activity in crude bovine thrombin fractionated as two distinct peaks, I and II. Crude bovine thrombin (7 mg, Calbiochem) was applied to a 1 ml HiTrap Q–sepharose column (Pharmacia) in 20 mM Tris–HCl, pH 8.0 and eluted with a linear 0 to 0.7 M NaCl gradient (upper dashed line) followed by a 1 M NaCl wash. Continuous line, protein profile (OD_{280}); closed squares with dashed line, activity profile quantitated as percentage of myotubes that were BrdU-positive. Peak I eluted at 200 mM NaCl, peak II at 440 mM NaCl. (c) The activity in peak I co-fractionated with thrombin. Peak I fractions from Q–sepharose were pooled, applied to a 1 ml HiTrap SP–sepharose column in 20 mM HEPES pH 7.5, 100 mM NaCl and eluted with a linear 0.1 to 0.7 M salt gradient (upper dashed line). The protein profile (continuous line) shows one major peak eluting at 500 mM NaCl. The activity profile (closed squares with dashed line) coincides with this peak. The pooled fractions of the major peak contained 280 μg/ml protein. *A silver-stained gel of the peak fraction following SDS–PAGE (12%) showed that it contained only the α, β and γ forms of thrombin (this was confirmed by western blot analysis using polyclonal antibodies to thrombin; data not shown). (d) Inhibition of thrombin proteolytic activity in the fraction eluted from SP–sepharose abolished its activity on myotubes. The SP–sepharose fraction was assayed at 19 μg/ml, and thrombin was present at approximately 0.5 μM. ATIII or hirudin was added to SP–sepharose fractions in a 2:1 or 5:1 molar excess, respectively, before assaying on myotubes. Control, SP–sepharose fraction with no inhibitor added.

Figure 2



Pure thrombin required the presence of serum to elicit cell-cycle re-entry. Thrombin (0.23 μM) was assayed on newt myotubes under serum-free (1% bovine serum albumin; BSA) conditions or in the presence of 1.5% foetal bovine serum. Thrombin elicited no response in serum-free conditions but had potent activity in 1.5% serum.

Figure 3



Thrombin- and plasmin-dependent proteolysis can generate S-phase re-entry activity from serum. (a) Medium containing 1.5% foetal bovine serum (which does not induce S-phase re-entry) was incubated with thrombin or plasmin for 24 h at room temperature before protease inhibitor treatment (hirudin or α2-antiplasmin) as described in the Materials and methods. This preparation was added to newt myotubes, which were then assayed for BrdU incorporation by a standard assay procedure and then fixed. In control experiments, protease and inhibitor were added simultaneously. The proteolytic activity in samples was monitored at 0 and 24 h (asterisks) using the chromogenic substrates tosyl-glycyl-prolyl-arginine-4-nitranilide acetate (ChromozymTH, Boehringer) for thrombin and tosyl-glycyl-prolyl-lysine-4-nitranilide acetate (ChromozymPL, Boehringer) for plasmin (see Materials and methods). (b) Thrombin-dependent generation of activity from 1.5% serum. Filled diamonds, samples incubated with thrombin for 24 h before protease inhibitor treatment; open squares, samples in which thrombin and hirudin were added simultaneously. Similar results were obtained using PPACK as inhibitor. (c) Plasmin-dependent generation of activity from 1.5% foetal bovine serum. Filled diamonds, samples incubated with plasmin for 24 h before protease inhibitor treatment; open squares, samples in which plasmin and α2-antiplasmin were added simultaneously. Although plasmin activity was largely neutralised by serum, α2-antiplasmin was used to inhibit any residual proteolytic activity. Other proteases that were tested in this assay and found to be negative included trypsin, Factor Xa, Protein Ca, Factor IX and Factor XII.

myotubes by amino-terminal cleavage and activation of its membrane receptor [26,27], or indirectly by cleavage of molecules in serum leading to activation or generation of a ligand which then acts directly on the myotubes through a pathway unrelated to the thrombin receptor. The newt myotubes were unresponsive to the thrombin-receptor agonist peptide (Table 1, legend), but more conclusive evidence for the second, indirect model has come from the use of thrombin inhibitors.

Thrombin generates re-entry activity in serum

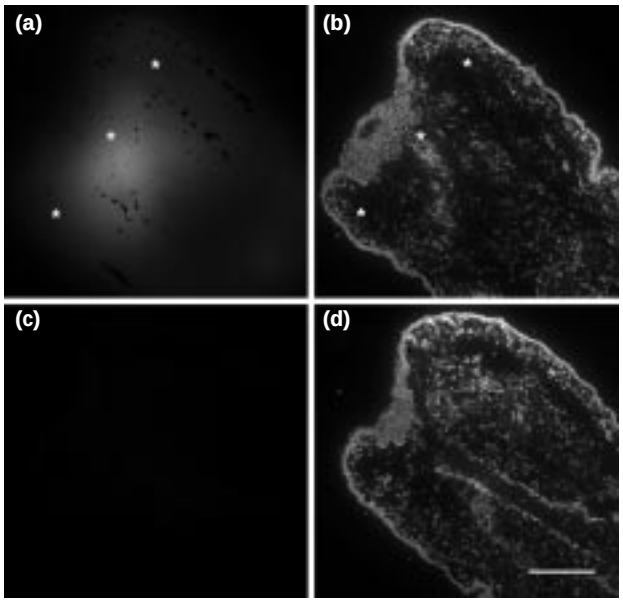
Medium containing a sub-threshold amount of serum was pre-incubated with thrombin for 24 hours, before addition of sufficient hirudin or PPACK to inactivate protease activity (Figure 3a and see Materials and methods). The resulting medium was added to the newt myotubes in a standard assay. The thrombin pre-incubation generated significant re-entry activity, up to a thirtyfold increase over the basal level in the medium, and this was dependent on the thrombin concentration (Figure 3b). In control experiments, thrombin and its inhibitor were added together at the beginning of the preincubation, and this generated no significant re-entry activity (Figure 3b).

A variety of proteases were tested in this serum-generation assay (Figure 3, legend), but these were inactive with one exception. When plasmin was incubated as shown in Figure 3a and then inactivated with α2-antiplasmin, it generated significant activity on the newt myotubes, and this activity was not detected when the inhibitor and protease were added simultaneously (Figure 3c). Thus, the two coagulation-related proteases were able to generate re-entry activity from components in serum.

Thrombin activity is elevated in the limb blastema

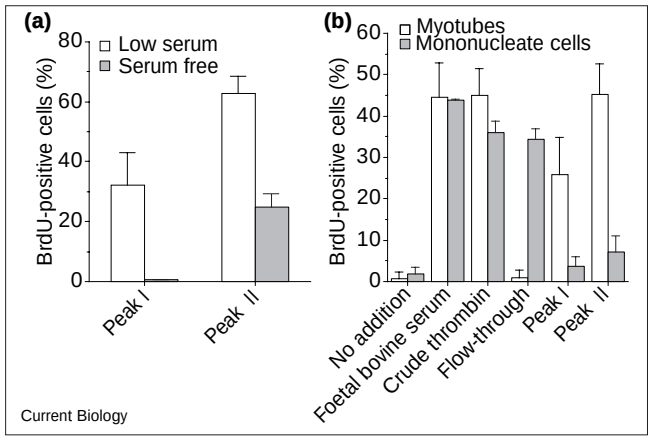
To evaluate the possible significance of this indirect mechanism for inducing cell-cycle re-entry during regeneration, we assayed thrombin activity in the blastema with an overlay method which permits tissue-level resolution [28]. Newt limbs undergoing regeneration were sectioned in a cryostat 8 days after amputation, a stage when the blastema is forming by reversal of differentiation in the

Figure 4



Thrombin proteolytic activity is elevated in the zone of de-differentiation. Thrombin proteolytic activity was localised in sections of regenerating newt limbs using a membrane overlay procedure. **(a,c)** Membrane overlay assay on a section through a day 8 regenerate in the (a) absence or (c) presence of the thrombin inhibitor PPACK. In (a), there is a strong fluorescent reaction product in the mesenchymal tissue at the end of the stump. No fluorescent signal is detectable in (c). **(b,d)** Propidium iodide staining of the identical sections shown in (a,c), respectively, to show nuclei within the regenerate. The scalebar represents 1 mm.

Figure 5



Evidence that the Q–sepharose peak II (see Figure 1) contains the activity generated downstream of thrombin that acts directly on myotubes. **(a)** Activity of peaks I and II on myotubes cultured in low serum (0.5%) or serum-free (1% crystallised BSA) conditions. Peak I was assayed at 50 μg/ml, and peak II at 1 mg/ml protein concentration. Higher concentrations of peak I resulted in lower cell responses or cell detachment. Peak I, which contains purified thrombin, was active in low-serum conditions but not in serum-free conditions whereas peak II was active under both low-serum and serum-free conditions. **(b)** Activity of Q–sepharose peaks on myotubes versus mononucleate precursors. Samples from the peak fractions were added to myotubes in low-serum media, or, in parallel, to mononucleate cells that had been serum starved for 24 h and maintained in serum-free media. Although the crude thrombin preparation stimulated cell-cycle re-entry in both the myotubes and mononucleate cells, the chromatographic properties of the activities were distinct. The mitogenic activity for mononucleate cells was found in the Q–sepharose flow-through fraction, whereas peaks I and II had very low levels of such activity. In contrast, the activity that stimulates the newt myotubes was absent in the flow-through fraction, but high in peaks I and II. The following concentrations were employed for these assays: foetal bovine serum (9 mg/ml), crude thrombin (69 μg/ml), Q–sepharose flow-through (< 50 μg/ml), peak I (40 μg/ml), peak II (0.78 mg/ml).

mesenchymal tissue at the end of the stump. The sections were overlaid with a membrane impregnated with a fluorogenic thrombin substrate — a coumarin ester of D-Phe-Pro-Arg (see Materials and methods). This resulted in selective generation of fluorescent reaction product in the mesenchyme at the end of the limb underneath the wound epidermis (Figure 4a,b, asterisks). The signal was completely inhibited by inclusion of PPACK (Figure 4c,d). It is interesting that thrombin activity is locally high in this region at a time significantly later than the acute events of clotting, which occur immediately after amputation. As this mechanism might operate during limb regeneration, and during local wounding (see Discussion), it was important to study the ligand that is generated downstream of thrombin activation, and that acts directly on the myotube.

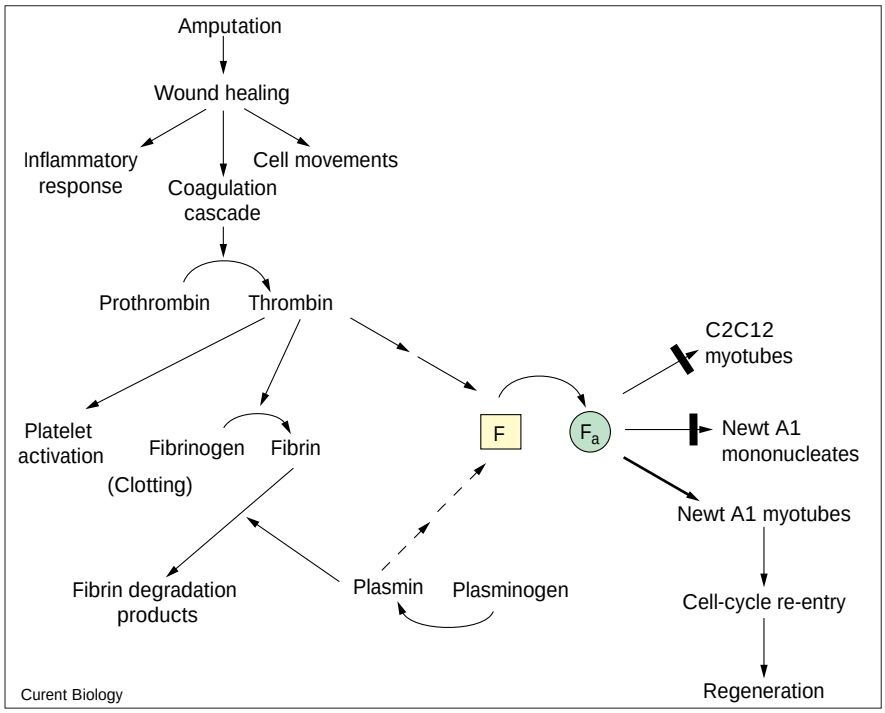
Identification of a second re-entry activity

We analysed the second activity detected in crude thrombin preparations (Figure 1b), which appeared to have the properties expected of the ligand in question. When peak I, previously identified as thrombin (Figure 1c,d), was assayed in the presence and absence of serum, it was found to be completely serum dependent (Figure 5a) as observed for purified thrombin (Figure 2). In contrast, peak II, which eluted from Q–sepharose at 440 mM NaCl, retained approximately 40% of its activity on myotubes under serum-free conditions (Figure 5a). Preparations treated with PPACK to remove all traces of thrombin activity also retained significant activity in serum-free media.

A notable feature of peak II is its profile of activity on mononucleate cells versus myotubes (Figure 5b). Whereas foetal bovine serum and crude thrombin were active, as expected, on both myotubes and mononucleate cells, the activities in peaks I and II had little or no effect on mononucleate cells. The activity on mononucleate cells, which is present in the crude thrombin preparation, is a distinct component which flows through the Q–sepharose column and has a specificity resembling mitogenic growth factors (see Table 1) in that it acts on the precursors but not on the differentiated myotubes. Thus, the activity in peak II

Figure 6

Schematic diagram of the activation of S-phase re-entry by newt myotubes in the context of the wound-healing responses that lead to regeneration. Amputation of the limb triggers multiple responses such as inflammation and cell migration. A major aspect of wound healing is activation of the coagulation cascade, resulting in the conversion of prothrombin to thrombin. In mammals, this induces the conversion of fibrinogen to fibrin polymers and formation of a clot. In newts, thrombin activation, in addition, leads to cell-cycle re-entry from the differentiated state. This involves the conversion of a latent activity (F to Fa) within serum which can selectively stimulate newt myotubes, but not their mononucleate precursors, to undergo S phase. Although the Fa activity was found in all animal sera tested, and hence is likely to be a general product of thrombin activation, mouse C2C12 myotubes were refractory to this activity.



appeared to act specifically on myotube S-phase re-entry. Thrombin-treated serum preparations (as in Figure 3b) also showed the same selective activity on myotubes but not on mononucleate cells (data not shown). Although firm conclusions must await the purification and identification of the activity in peak II, it is also notable that this activity has the same elution profile after gel filtration on Superose 12 as the activity in serum (Mr = 2.5×10^5).

Discussion

In earlier work, we found that phosphorylation of Rb, an intracellular regulator of the cell cycle, appeared to be necessary to induce S-phase re-entry by newt myotubes [11]. The present results indicate that this pathway is initiated by the generation of a distinct extracellular activity. The appearance of this activity *in vitro* could be regulated by thrombin-mediated proteolysis, and also by plasmin, whereas various other proteases were negative in this assay. It is unclear whether the mechanism by which this activity is generated involves direct cleavage of a precursor form, cleavage and inactivation of an inhibitor, or initiation of a more complex cascade. Nonetheless, our results provide a connection between plasticity of the differentiated state and the acute events of injury, wound healing and haemostasis [29,30]. In this context, it is interesting that thrombin activity was found to be elevated in the mesenchymal tissue at the end of the limb stump, at a stage when both implanted A1 myotubes and endogenous myofibres have been observed to enter S phase (A. Kumar, C. Velloso, Y. Imokawa and J.P.B., unpublished observations).

It has long been recognised that local wounding of the urodele limb or flank evokes the appearance of various markers characteristic of blastemal cells [31], including two members of the HoxD complex [32]; furthermore, if a major peripheral nerve is transected and inserted into such a wound so as to stimulate division, then a supernumerary limb may form [33]. We suggest that local activation of thrombin may be an important signal for cell-cycle re-entry by differentiated cells through the indirect mechanism identified here (see Figure 6 for summary). It is obviously important that de-differentiation remains localised to the zone at the end of the stump, just as it is critical that formation of a clot does not propagate from the site of injury [29]. Therefore, the mechanisms that localise and inactivate thrombin in the latter case may also be relevant to the former.

The identity of the ligand that is generated as a downstream product of thrombin action is currently not known. It clearly does not involve further thrombin activity, and can be separated from inactivated thrombin by subsequent chromatography. It is unlikely to involve further protease activity given that it is resistant to broad spectrum serine-protease inhibitors. The identification of angiogenic and anti-angiogenic activity in fragments of fibrin, collagen XVIII and plasminogen raises the possibility that other

products of proteolysis may carry biological activity [34–36]. We have investigated several substrates of thrombin in serum, including protein C, fibrinogen/fibrin, apolipoprotein E and thrombospondin, but none have led to activity on newt myotubes. Although characterisation of the active species will require significant further purification, the activity as identified has several interesting properties. Not only does it act, unlike thrombin, in serum-free medium, but it also shows specificity for the myotube as distinct from mononucleate cells — the opposite distinction to that made by mitogenic growth factors such as PDGF. It is likely, therefore, that responsiveness is a specific property of the differentiated state; it is clear that the division of mononucleate blastemal cells, once they are generated, is regulated by quite different mechanisms involving local positional disparity as well as the nerve supply [7,37,38].

Finally, our results emphasise the importance for regeneration of the difference in responsiveness between newt myotubes and their mammalian counterparts, rather than the existence of any urodele-specific signal. The activity referred to as Fa (Figure 6) was detected in all sources of serum that we have analysed, and its generation in vertebrates may be a universal feature of the downstream events following wounding and thrombin activation. In this view the regenerative ability of the newt depends critically on the responsiveness of its differentiated cells, as exemplified here by the cultured and implanted myotube. The characterisation of Fa should allow an analysis of the pathway leading to phosphorylation of Rb in the myotube, and eventually to an understanding of why it does not operate in the mammalian myotube, and whether it operates in any other mammalian cells.

Materials and methods

Cell culture and myotube purification

Newt A1 cells were cultured essentially as described [9,11]. Cells were grown on gelatin-coated plastic in 65% Eagles MEM, 10% heat-inactivated foetal bovine serum, 25% H_2O, 10 μg/ml insulin and penicillin/streptomycin. For myotube purification, two plates of cells, each containing 8×10^5 cells in medium containing 0.5% serum (low-serum medium), were cultured for 4 days to induce myogenic differentiation. The cells were then trypsinised, neutralised in 0.5% serum-containing medium, filtered through 100 μm meshes (Cell MicroSieve, BioDesing Inc.) and the filtrate passed through 35 μm meshes. The myotubes retained on the 35 μm meshes were washed into low-serum medium and plated into a gelatin-coated 96-well plate (150 μl/well).

Substances such as growth factors were added 24 h after plating and samples were always assayed in triplicate and the results averaged. Unless otherwise specified, cells were routinely assayed in the presence of 1.5% foetal bovine serum, which gave a response of < 1.5% BrdU-positive myotubes in an assay for which the maximal response was always at least 35% BrdU-positive myotubes. For the serum-free assays, myotubes were purified and plated in media with serum substituted by 1% BSA (final), and assayed. BrdU was added at 10 μg/ml on day 4 for 18 h. Cells were fixed briefly in 2% paraformaldehyde and rinsed twice in 0.1% BSA/PBS before post-fixing with methanol. Cells were stained for BrdU as described previously [11] using a double layer of secondary antibodies: rhodamine-conjugated rabbit anti-mouse IgG, and then rhodamine-conjugated swine anti-rabbit IgG (Dakopatts). Myotubes were then stained directly for muscle-specific myosin heavy chain using monoclonal mouse IgG A4.1025 (a kind gift of S. Hughes) which had been coupled to Fluorescein-NHS (Molecular Probes).

C2C12 cells were propagated essentially as described [11]. They were grown in 15% foetal bovine serum in DMEM with 10 μg/ml insulin. Myotube differentiation in a confluent 10 cm plate was induced by switching to medium containing 2% horse serum. C2C12 myotubes were purified as described above for newt A1 moytubes except cells were passed through the 35 μm mesh and trapped onto a 15 μm mesh.

Growth factors

The following kindly provided growth factors: Ontogeny (sonic hedgehog), C. Heldin (platelet lysate), C. Hill (activin A), E.J. Leonard (MSP), A. Ridley (HGF), L. Cheng and A. Mudge (GGF), and M. Noble (bFGF, EGF, IGF-1). KGF and TNF-α were from R+D laboratories; and retinoic acid, lysophosphatidic acid and thrombin-receptor agonist peptide were from Sigma; mCSF and TGF-β2 were from Genzyme.

Protease treatment of serum

All purified proteases and bovine antithrombin III were purchased from Enzyme Research Laboratories except trypsin, which was purchased from GIBCO. Hirudin and D-Phe-Pro-Arg chloromethyl ketone (PPACK) were purchased from Calbiochem, and α2-antiplasmin was from Sigma.

For generation of activity in serum by thrombin or plasmin, 1.5% serum-containing medium was incubated with thrombin (0.23–0.92 μM) or plasmin (0.09–0.53 μM) for 24 h at 25°C in 2% CO_2. Proteolytic activity in samples was monitored just after addition and at 24 h using the chromogenic substrates tosyl-glycl-prolyl-arginine-4-nitranilide acetate (ChromozymTH, Boehringer) for thrombin, and tosyl-glycyl-prolyl-lysine-4-nitranilide acetate (ChromozymPL, Boehringer) for plasmin. Samples were monitored at 405 nm every 20 s for at least 3 min to determine activity. In uninhibited samples, approximately 70% of the thrombin activity remained, whereas, at most, 50% of plasmin activity remained after 24 h. To inhibit the remaining proteolytic activity, hirudin was added at a 5:1 molar excess in thrombin experiments, and α2-antiplasmin at a 2:1 molar excess in plasmin experiments. This resulted in most cases in undetectable levels of activity, and in all cases proteolytic activity was inhibited to at least fourfold less than the levels required to elicit any BrdU uptake in myotubes; 100 μl of inhibitor-treated sample was then added to each well for assay.

Fractionation of crude thrombin

Q–sepharose fractionation of crude thrombin (Figure 1b) was performed using FPLC (Pharmacia). Crude thrombin (0.8 ml, 7 mg/ml, 800 NIH units; Calbiochem) was loaded onto a 1 ml HiTrap Q column (Pharmacia) in 20 mM Tris-HCl pH 8.0 at a flow rate of 1 ml/min. Non-adherent protein was washed in five column volumes and proteins were eluted with a 0–700 mM NaCl gradient over 10 column volumes. Fractions of 1.25 ml were collected and 1 ml of each was desalted into serum-free cell culture medium using NAP-10 columns (Pharmacia). Protein was monitored by absorbance at 280 nm and in pooled fractions using BCA protein determination reagent (Pierce).

For purification of thrombin (Figure 1c), 0.8 ml crude thrombin (7 mg/ml, 800 NIH units; Calbiochem) was loaded onto a 1 ml HiTrap Q column (Pharmacia) in 20 mM Tris-HCl, 100 mM NaCl pH 8.0 at 1 ml/min. Under these conditions, peak I (containing thrombin) was not retained on the column and flowed through. Non-adherent protein was washed in five column volumes and proteins were eluted with a 100–600 mM NaCl gradient over 10 column volumes. The flow-through fraction (containing peak I) from the HiTrap Q column was pooled and 2.5 ml was desalted into 20 mM HEPES pH 7.5, 100 mM NaCl on a PD10 column (Pharmacia); 3.5 ml of the desalted material was loaded onto a 1 ml HiTrap SP column (Pharmacia) in 20 mM HEPES pH 7.5, 100 mM NaCl at 1 ml/min. Non-adherent protein was washed in five column volumes and proteins were eluted with a 0–700 mM NaCl gradient over ten column volumes. Fractions (1.25 ml) were collected and 1 ml of each was desalted into newt

serum-free cell culture medium using NAP-10 columns (Pharmacia). The major peak eluted at 500 mM NaCl (Figure 1c).

All chromatographic fractions and pools were assayed at three or more protein levels covering a 20-fold range in concentration. Pools from Q–sepharose fractionation shown in Figure 5 have the following protein concentrations: Figure 5a – peak I, 0.37 mg/ml; peak II, 1.5 mg/ml; Figure 5b – foetal calf serum, 60 mg/ml; crude thrombin 6.9 mg/ml; flow-through, < 50 μg/ml; peak I, 0.3 mg/ml; peak II, 1.2 mg/ml.

Membrane overlay assays for thrombin

Localisation of thrombin activity in limb sections was performed as described [28]. Newt limbs at 8–15 days post-amputation were fast frozen on dry ice in Tissuetek (Sakura Finetek) and 10 μm sections were prepared, collected on silane-coated slides and stored at –80°C. Samples were air-dried at room temperature for 30 min, fixed in methanol, rehydrated in TBS, stained in 10 μm propidium iodide and then placed in TBS + 100 μg/ml BSA. Thrombin substrate membranes impregnated with 7-amino-4-trifluoromethyl coumarin-D-Phe-Pro-Arg (AFC-64, Enzyme System Products), were briefly hydrated in water, then TBS and placed immediately onto the sections. Excess liquid was removed and coverslips sealed in place with nail varnish. Images were collected between 1 and 5 hours later on a Zeiss Axiophot with a cooled CCD camera, and with computer software from Image Pro Plus (Photonic Science). Control sections were incubated with 1 μM PPACK before exposure to membrane. Images of control and experimental samples were collected under identical conditions. Nuclei within the sections were visualised under fluorescence optics after staining with propidium iodide.

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