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Mechanisms of Thrombin-Stimulated Cell Division

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Addition of highly purified thrombin to cultures of several kinds of nondividing fibroblasts brings about cell division. This stimulation occurs in serum-free medium, permitting studies on its mechanism under chemically defined conditions. Previous studies have shown that action of thrombin at the cell surface is sufficient to cause cell division and that the proteolytic activity of thrombin is required for its mitogenic effect. These results prompted experiments which showed that there is a cell surface receptor for thrombin and that thrombin must bind to its receptor and cleave it to stimulate cell division. Some of the thrombin that binds to its receptors becomes attached to them by a linkage that appears to be covalent. However, it is presently unknown whether this direct thrombin receptor complex plays a role in the stimulation.

These results raise a number of questions that should be explored in future studies. They also provide a foundation on which to build hypotheses about tentative molecular mechanisms that might be involved in the stimulation. Knowledge that thrombin must cleave its receptor to bring about cell division suggests two alternative mechanisms for stimulation by proteolysis. In one the receptor is a negative effector which prevents cell division when it is intact, but not after it has been cleaved. Alternatively, a fragment of the receptor could be a positive effector. In this mechanism, proteolysis by thrombin would produce a specific receptor fragment which brings about cell proliferation. If every protease which cleaves the receptor also stimulates cell division, the receptor is probably a negative effector. In contrast, if certain proteases cleave the receptor but do not stimulate the cells, a fragment of the receptor is likely a positive effector. With negative regulation by the receptor, the controlling events would occur before proteolysis of it, and it might be possible to find putative regulatory molecules by identification of nearest neighbors of the receptor. This should be possible by using bifunctional crosslinking reagents. If a fragment of the thrombin receptor turns out to be a positive effector, it should be possible to identify and study fragments by analyzing the metabolic fate of the receptor. Techniques are now available for this kind of analysis and it should also be possible to determine whether receptor fragments remain in the membrane or whether they are translocated to specific sites within the cell. A

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critical question to be asked is which of these events and interactions involving the thrombin receptor are necessary for stimulation of cell division. It now appears that the best way to answer this question is to examine these events in a large number of cloned cell populations that are responsive or unresponsive to the mitogenic action of thrombin. If a thrombin-mediated event occurs in all responsive clones but is altered or absent in some unresponsive clones, it is probably necessary for stimulation of cell division.

Key words: cell surface receptors, proteolysis of receptors, positive or negative regulation

Recent studies have shown that thrombin is an effective mitogen for several kinds of cultured fibroblasts. Addition of it to nondividing cultures of chick embryo (CE), mouse embryo (ME), or human foreskin (HF) cells causes about one round of cell division. Under optimal conditions, this treatment leads to a 60–80% increase in cell number with CE and ME cells and about a 40% increase with HF cells [1–3].

Investigations on the mechanism of thrombin-stimulated cell division have been aided by several properties of the system. First, cell division can be brought about by adding highly purified thrombin to serum-free cultures. Although factors normally present in the animal are absent, this system permits experiments to be conducted under chemically defined conditions. Second, the proteolytic activity of thrombin is required for its mitogenic action [2, 4], indicating that proteolysis is a primary event in the stimulation. Third, it has been possible to derive cells that are unresponsive to the mitogenic action of thrombin from responsive populations of cells [3]. By studying thrombin-mediated events in both responsive and unresponsive populations, it should be possible to determine which events are necessary for thrombin-stimulated cell division and which are not [5]. Finally, thrombin action at the cell surface is sufficient to bring about cell division [6]. Knowledge of this property of the system has facilitated studies on it by identifying a limited part of the cell on which to focus initial experiments.

In the first section of this article we will briefly summarize and discuss studies to date on thrombin-stimulated cell division, emphasizing results which have provided clues about molecular events involved. This will provide a framework for the second section where we will extrapolate to more detailed molecular mechanisms which are necessarily hypothetical at this time. Here, the emphasis will be on models and mechanisms which are amenable to experimental evaluation. We have recently published a summary of our work on thrombin-stimulated cell division which contains supporting experimental data [7].

REVIEW OF THROMBIN-STIMULATED CELL DIVISION

Action of Thrombin at the Cell Surface is Sufficient to Stimulate

We decided that the first goal in probing the mechanism of the stimulation should be identification of the cellular site(s) where the primary interactions with thrombin take place. The first specific question was whether thrombin could stimulate by acting at the cell surface or whether it had to be internalized. This question has been asked previously for other polypeptide growth factors and hormones, but the answers have not been conclusive. Early studies with polypeptides linked to Sepharose beads indicated that action at the cell surface was sufficient to elicit a biological response [8–11]. However, subsequent experiments showed that there was sufficient release of polypeptides from these

Sepharose beads [12–15] (sometimes in a "superactive" form [16]) to question this conclusion. It has also been questioned on the basis of extensive uptake of polypeptide factors by cultured cells [17–19], and demonstrations of receptors for insulin [20] and nerve growth factor [21, 22] in the nucleus.

We approached this question for thrombin by linking it to carboxylate-modified polystyrene beads via a peptide bond using a water-soluble carbodiimide reagent [6]. These thrombin beads stimulated division of cells; however, beads with nonmitogenic proteins like bovine serum albumin or ovalbumin similarly linked to them did not cause the cells to divide. The critical question, of course, was whether the stimulation by thrombin beads could be accounted for by release of thrombin from the beads. Carefully controlled experiments employing ^{125}I-thrombin linked to polystyrene beads demonstrated that there was not sufficient release either into the medium or directly into cells to account for any of the cell division caused by the thrombin beads. This permitted us to conclude that action of thrombin at the cell surface was sufficient to stimulate cells to divide [6]. Analogous experiments with trypsin, another protease that is mitogenic for CE cells, demonstrated that it also can produce cell division by action at the cell surface [23].

Binding of Thrombin to Its Cell Surface Receptors is Necessary for Stimulation

The above results indicated that it would be fruitful to examine the cell surface for specific interactions with thrombin. Binding studies with ^{125}I-thrombin, in the absence and presence of a large excess of unlabeled thrombin, showed that ME, HF, and CE cells bind thrombin specifically, indicating the presence of receptors for thrombin [24, 25]. The fraction of total binding that was specific was greater for ME cells than CE or HF cells so we have conducted most of our binding experiments with ME cells. The receptors on ME cells appear to be unique for thrombin, since neither insulin nor epidermal growth factor (EGF) compete significantly for the binding of ^{125}I-thrombin. The thrombin receptor is actually on the cell surface, since about 80% of ^{125}I-thrombin specifically bound under steady state conditions can be removed by brief treatments with trypsin which do not disrupt cells. Scatchard analyses of ^{125}I-thrombin binding data are linear over a broad range of thrombin concentrations, indicating a single affinity class of receptors [24].

The conclusion that thrombin must bind to its receptors on ME cells to stimulate cell division came from experiments in which small amounts of calf serum were added along with thrombin. Addition of calf serum to a final concentration of 0.1% markedly inhibited stimulation of ME cell division by thrombin. This low concentration of serum markedly inhibited the binding of thrombin to its cell surface receptors, but it did not inhibit either the proteolytic activity of thrombin or its nonspecific association with the cells. Scatchard analyses of ^{125}I-thrombin binding data in the presence and absence of serum showed that the inhibition by serum resulted from a masking of thrombin receptors on cells and not from binding of ^{125}I-thrombin by serum components [24].

Photoaffinity Labeling of the Thrombin Receptor

The indication, noted above, that there was a single affinity class of thrombin receptors suggested the possibility that they could be identified as a discrete molecular species by photoaffinity labeling techniques. By employing procedures developed by Fox and his collaborators to radiolabel the EGF receptors on mouse 3T3 cells [26, 27], we labeled the thrombin receptors on ME [28] and CE [5] cells with ^{125}I-thrombin con-

jugated to a photoreactive reagent. These experiments revealed that there was significant labeling of what appeared to be only a single component or receptor on the cell surface. The apparent molecular weight of the receptor was about 50,000 for ME cells, and 43,000 for CE cells. The absolute values of these molecular weights must be interpreted with caution since the receptors might be glycoproteins. Nevertheless, the ability to identify the labeled thrombin-receptor complex on gels has enabled us to make some interesting conclusions.

Thrombin Must Cleave Its Receptors to Stimulate Cell Division

A question that is very basic to the mechanism of stimulation by thrombin is whether its proteolytic activity is required for mitogenic action. It appears that proteolysis is indeed necessary, since thrombin that has been inactivated with either diisopropyl-fluorophosphate (DFP) or phenyl methyl sulfonyl fluoride (PMSF) is no longer mitogenic even though these inactivated thrombins bind to cells like active thrombin [4]. This requirement for proteolysis prompted us to search for cell surface components whose cleavage by thrombin was necessary for stimulation of cell division. We did this by looking for cell surface components that were cleaved by thrombin on CE cells that were responsive to its mitogenic action, but which were not cleaved or were absent on CE cells that did not divide after thrombin treatment [5]. Cleavage of cell surface proteins was monitored by labeling cell surface components of control and thrombin-treated cells with $^{125}I^-$ by lactoperoxidase-catalyzed iodination. These studies revealed a cell surface component of 43,000 daltons (43k) that was removed by thrombin from the responsive cells. An apparently identical component was present on four separately isolated populations of unresponsive cells, but it was not removed by mitogenic treatments with thrombin. Thus, removal of it appears necessary for thrombin-stimulated cell division. It is noteworthy that 43k was not removed from responsive cells by DFP-inactivated thrombin. This shows that proteolysis by thrombin and not a cellular protease was responsible for its removal. It was also not removed during serum stimulation, demonstrating that its removal was not simply a consequence of initiating cell division [5].

As noted above, the photoaffinity labeling experiments showed that the molecular weight of the thrombin receptor on CE cells was 43,000. Because of the identity of their apparent molecular weights, it appears that 43k, identified by labeling with $^{125}I^-$ and lactoperoxidase, is the thrombin receptor. It should be pointed out that there has been some variability in the migration on sodium dodecyl sulfate (SDS) polyacrylamide gels of the thrombin receptor complex of CE cells from experiment to experiment, depending on the condition used to solubilize the cells. However, there was a corresponding variation in the migration of ^{125}I-thrombin so the estimated molecular weight of the thrombin receptor remains at about 43,000. Thus, the studies to date indicate that thrombin must cleave its receptor to stimulate cell division although this conclusion is based on measurements of molecular weight that necessarily involve some uncertainty. It is noteworthy that the unresponsive cells still specifically bound ^{125}I-thrombin, consistent with the observation that 43k was still present on their cell surface. Thus, their inability to divide after thrombin treatment can be attributed to an absence of cleavage of their receptors rather than an inability of the receptors to bind thrombin.

Direct Linkage of Thrombin to Its Cell Surface Receptors

Another event has been identified which is a consequence of interaction between thrombin and its cellular receptors. When ^{125}I-thrombin is incubated with HF, ME, or

CE cells in the absence of any crosslinking reagent, a significant amount of the thrombin that is specifically bound becomes directly linked to its receptors [29, 30]. For HF cells, this amount is over 50%; it is about 1–10% for ME and CE cells. This linkage appears to be covalent since it survives boiling in 3% SDS and 1.0% 2-mercaptoethanol.

It is noteworthy that the linkage can be disrupted by incubation at pH 12 or treatment with hydroxylamine [30]. This suggests that the linkage between thrombin and its receptors involves an acyl group, analogous to the linkage that occurs between thrombin and antithrombin III [31, 32]. This ester linkage is formed between an arginine carboxyl group of antithrombin III, and the hydroxyl group of the active site serine of thrombin, representing a stable intermediate of a proteolytic event [31]. In this case, the released peptide fragment has a molecular weight of about 6,000 since the apparent molecular weight of the thrombin-antithrombin III complex under reducing conditions is about 90,000, while that of antithrombin III is about 63,000, and thrombin is about 33,000 [33]. Our preliminary experiments are consistent with the possibility that formation of the direct thrombin receptor complex is analogous to formation of the thrombin-antithrombin III complex, and that only a small peptide from the receptor is released upon complex formation. Clearly, there are many questions about the thrombin receptor complex that remain to be answered. If it turns out to be a stable intermediate of a proteolytic reaction, like the thrombin-antithrombin III complex, it will be important to investigate any relationship it might have to the cleavage of the thrombin receptor identified by experiments discussed in the preceding section [5].

POSSIBLE MECHANISMS FOR THROMBIN-STIMULATED CELL DIVISION

The above results suggest some likely models or mechanisms for the stimulation by thrombin. They also raise a number of questions that should be addressed in future studies. There are many possibilities, and in this section we will discuss some testable alternatives that are providing direction to our experiments.

Purification of the Thrombin Receptor

Many studies on the mechanism by which the receptor participates in stimulation of cell division would be greatly facilitated by a purified receptor and antibodies to it. For example, availability of a purified receptor would permit a direct test of whether the receptor is identical to the 43,000 dalton cell surface component identified by iodination with $^{125}I^-$ and lactoperoxidase. We are currently developing several purification procedures. One of these involves incubating cells with ^{125}I-thrombin and purifying the directly linked ^{125}I-thrombin receptor complex. With this method, the assay during purification simply involves measurements of ^{125}I-radioactivity, and the receptor can be liberated from the ^{125}I-thrombin receptor complex after purification by treatment with hydroxylamine or pH 12 as noted above. Our preliminary experiments indicate that human placenta will be useful for this purification since it forms a large amount of the ^{125}I-thrombin receptor complex upon incubation with ^{125}I-thrombin. We have found that the complex binds to heparin with a high affinity, and this has provided the basis for an effective affinity purification step. In addition, the ^{125}I-thrombin receptor complex can be precipitated by antibodies to thrombin, and this should facilitate the development of a second affinity purification step. Combined with standard fractionation techniques, these highly selective purifications should enable us to extensively purify the thrombin receptor complex, and thus the receptor.

We are also developing procedures to directly purify the receptor itself. Our experiments have shown that it binds with high affinity to heparin, and this should make it possible to develop an affinity purification step as with the thrombin receptor complex. It will be interesting to compare the directly purified receptor with the receptor that is liberated by hydroxylamine or pH 12 treatment from the purified ^{125}I-thrombin receptor complex. As noted earlier, formation of this complex probably involves removal of a small fragment from the receptor. It will be possible to study this problem directly when both receptor preparations have been purified.

The Thrombin Receptor as a Positive or Negative Effector

The conclusion that thrombin must cleave its receptor to stimulate cell division suggests two alternative mechanisms by which proteolysis could trigger cell division. One possibility is that the thrombin receptor is a negative effector which in its intact state participates in the development of a negative signal to prevent cell division. The other possibility is that cleavage of the receptor by thrombin produces a specific receptor fragment which is a positive effector and signals the cell to divide. It should be possible to distinguish between these two possibilities by examining the ability of a number of different proteases to stimulate cell division and cleave the thrombin receptor. If every protease which cleaves the receptor also stimulates the cell to divide, it would appear that the receptor functions as a negative effector. On the other hand, if there are proteases which cleave the receptor but do not stimulate cell division, it is likely that proteolysis of the receptor by thrombin produces a specific positive fragment which is central to the development of the mitogenic signal.

It is noteworthy that thrombin must be present continuously in the medium to produce maximal cell division, even though a one hour exposure to it removes its receptors from the cell surface [5]. However, the receptors are replaced within three hours after changing to thrombin-free medium [5]. Therefore, if the thrombin receptor is a negative effector, it must be removed continuously to allow maximal cell division; replacement of it would stop events leading to cell proliferation. On the other hand, if a specific fragment of the receptor is a positive effector, the above results predict it would have a short half-life. As evidence becomes available for either alternative, it will be important to develop approaches to test these predictions.

Approaches for Studying Negative Regulation by the Receptor

There are several possible mechanisms which could account for negative regulation where the thrombin receptor prevents cell division when it is intact but not after it has been cleaved by any one of several proteases. For example, the intact receptor might interact with and inhibit a membrane enzyme necessary for production of a product that is rate limiting for cell division; cleavage of the receptor would prevent inhibition of enzymatic activity and lead to stimulation of cell division. Alternatively, the intact receptor, but not a cleaved receptor, might bind a cytoplasmic agent which permits or causes cell division when free but not when bound to the receptor. Such an agent could be a component of the structural or contractile apparatus, an ion, or a specific positive regulatory molecule. There are, of course, many possibilities. The important point is that with negative regulation by the thrombin receptor, the key controlling events would occur before proteolysis of it. Thus, if the experiments in the preceding section indicate that the receptor is a negative effector, it would be furitful to identify cellular components that are bound to the thrombin receptor and to determine if proteolysis of the receptor

affects this association. Protein-nearest neighbors of the receptor could be identified by previously developed techniques employing a reversible bifunctional imidoester cross-linking reagent, immunoadsorption with antibodies to thrombin, and two-dimensional diagonal electrophoresis [34–36]. This approach would facilitate identification of putative regulatory components that are bound to the thrombin receptor in its intact state but not after proteolytic cleavage of it.

Approaches for Studying Positive Regulation by the Receptor

If certain proteases can cleave the thrombin receptor without producing cell division, it is likely that stimulation by thrombin involves production of a specific receptor fragment which is a positive effector. In view of the very limited and specific proteolysis by thrombin of protein substrates [37], such a mechanism is certainly feasible.

From our present perspective, it appears that positive regulation by a fragment of the receptor would be easier to explore than negative regulation by the intact receptor. This is largely because a first step in examining positive regulation would be a study of the metabolic fate of the receptor, and it is now possible to propose feasible approaches for this. One avenue has been provided by model studies on the EGF receptor involving linkage of ^{125}I-EGF to its receptors by a photoactivable cross-linking reagent [26, 27]. Since the crosslinked ^{125}I-EGF receptor complex was the only labeled component in the cells, it was possible to examine the cellular processing of it during continued incubation of the cells [27]. We have used analogous techniques to label the thrombin receptor on ME [28] and CE [5] cells with ^{125}I-thrombin conjugated to a photoactivable crosslinking reagent, and this should permit analysis of the metabolic fate of the thrombin receptor.

As noted above, we recently found that ^{125}I-thrombin in the absence of a cross-linking reagent becomes directly linked to its cellular receptors [29, 30]. By following the fate of this linked component during continued incubation of cells, it should be possible to evaluate the metabolic fate of the spontaneously-formed direct thrombin receptor complex. In these studies, it will be important to determine if linkage of ^{125}I-thrombin to its receptor might alter subsequent processing of it. Only a fraction of the receptors that bind thrombin become crosslinked to it, and the subsequent metabolic fate of linked and unlinked receptors might be different. However, studies on the EGF receptor have shown that the same discrete fragments are produced after EGF binding whether or not EGF is crosslinked to the receptor. This conclusion came from experiments showing that the fragments derived from the EGF receptor were the same whether the linkage with the photoactivable crosslinking reagent was brought about before or after processing of the receptor [27]. In addition, when cells are incubated with ^{125}I-EGF, some of the specifically bound ^{125}I-EGF becomes directly linked to its receptors as does thrombin [29, 38]. Upon continued incubation, this complex is processed to fragments that are identical in size to the fragments identified with the photoactivable derivative of ^{125}I-EGF [38].

Preliminary experiments suggest that a portion of the direct ^{125}I-thrombin receptor complex is removed upon continued incubation of cells. After this incubation and treatment at pH 12 to disrupt the linkage between ^{125}I-thrombin and its receptor, the ^{125}I-radioactivity migrated with intact thrombin, indicating that a part of the receptor rather than a part of the thrombin had been removed from the thrombin receptor complex. Since the thrombin remained intact during the metabolism of the thrombin receptor complex, it should be possible to immunoprecipitate the metabolized thrombin receptor complex from NP40-solubilized extracts of ^{35}S-methionine-labeled cells using antibodies

to thrombin. Treatment of the immunoprecipitate at pH 12 should then release the ^{35}S-labeled receptor fragment from the metabolized thrombin receptor complex. This labeled fragment would be useful for many studies.

Determination of Which Events Are Necessary for Thrombin-Stimulated Cell Division

Since thrombin must bind to its cellular receptor and cleave it to stimulate cell division [5, 24], a first step in analyzing the mechanism of thrombin stimulation is to identify and define molecular changes in the thrombin receptor after thrombin binds to it. Then, it is important to evaluate which of these events are necessary for the stimulation and which are not.

An approach which we are refining is to examine these events in cells that are responsive and ones which are unresponsive to the mitogenic action of thrombin [3, 5]. Thrombin-mediated events which occur in all of the responsive populations but which do not occur or are altered in some of the unresponsive populations are probably necessary for the stimulation. We have conducted some of our experiments on uncloned populations of responsive and unresponsive CE cells [5]. We are now developing procedures to select and clone cells that are responsive or unresponsive to thrombin so it will be possible to conduct experiments on homogenous populations of cells. Since these experiments necessarily require cells that are capable of undergoing a large number of population doublings in culture, we are focusing attention on established cell lines. Preliminary experiments indicate that a line of Chinese hamster lung cells will be well suited for these studies. The above approach should enable us to address important issues. For example, a central question now is whether formation of the direct thrombin receptor complex is necessary for thrombin-stimulated cell division. There are indications that it might not be necessary since ME and CE cells are more sensitive to the mitogenic action of thrombin than HF cells, yet formation of the direct thrombin receptor complex is much more extensive with HF cells [30]. On the other hand, certain results suggest a correlation between formation of the complex and stimulation by thrombin. The important point here is that the clearest means for resolving this issue will be examination of thrombin receptor complex formation in a large number of cloned responsive and unresponsive cells.

The approaches discussed in this section might enable us to define a metabolic pathway for the thrombin receptor that is involved in the control of cell proliferation. If these studies identify receptor fragments that are necessary for stimulation, it would be important to extend the above techniques and examine the fate of these fragments. Some might be further metabolized to species that are also necessary for stimulation. An active fragment could bring about its biological effects in the membrane of the cell surface or endocytosed vesicles, or it could be released from the membrane and produce its effects by interacting with a soluble cell component or with the nucleus or another cellular organelle. Thus, it would be important to examine the fate of these receptor fragments in terms of their location in the cell after continued incubation. This information probably could be obtained by EM autoradiography if the fate of the receptor were studied by following the fate of the ^{125}I-thrombin receptor complex. Then, responsive and unresponsive clones could be used to determine whether certain associations of receptor fragments with cellular components are necessary for thrombin-stimulated cell division.

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