The Mitogenic Effects of the Bβ Chain of Fibrinogen Are Mediated through Cell Surface Calreticulin*

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We have previously shown that soluble partially degraded fibrin(ogen) remains in solution after fibrin clot formation and is a potent fibroblast mitogen (Gray, A. J., Bishop, J. E., Reeves, J. T., Mecham, R. P., and Laurent, G. J. (1995) Am. J. Cell Mol. Biol. 12, 684–690). Mitogenic sites within the fibrin(ogen) molecule are located on the Aα and Bβ chains of the protein (Gray, A. J., Bishop, J. E., Reeves, J. T., and Laurent, G. J. (1993) J. Cell Sci. 104, 409–413). However, receptor pathways through which mitogenic effects are mediated are unknown. The present study sought to determine the nature of fibrin (ogen) receptors expressed on human fibroblasts which interact with the fibrinogen Bβ chain. Receptor complexes were isolated from 125I-surface-labeled fibroblasts and purified on a fibrinogen Bβ chain affinity column. Subsequent high performance liquid chromatography and SDS-polyacrylamide gel electrophoresis analysis indicated fibrinogen Bβ chain bound specifically to a 60-kDa surface protein. Sequence analysis of the amino terminus of this protein indicated 100% homology to human calreticulin. Immunoprecipitation experiments employing a polyclonal anti-calreticulin antibody provided further evidence that the 60-kDa protein isolated in this study was calreticulin. Further, polyclonal antibodies to human calreticulin significantly inhibited the mitogenic activity of fibrinogen Bβ chain on human fibroblasts. The present study has shown that cell surface calreticulin binds to the Bβ chain of fibrinogen mediating its mitogenic activity.

Blood coagulation at the site of tissue injury is a process central to wound healing. The deposition of an insoluble fibrin clot provides both a hemostatic plug preventing leakage of extracellular matrix and isapotent fibroblast mitogen (Gray, A. J., Bishop, J. E., Reeves, J. T., Mecham, R. P., and Laurent, G. J. (1995) Am. J. Cell Mol. Biol. 12, 684–690). Mitogenic sites within the fibrin(ogen) molecule are located on the Aα and Bβ chains of the protein (Gray, A. J., Bishop, J. E., Reeves, J. T., and Laurent, G. J. (1993) J. Cell Sci. 104, 409–413). However, receptor pathways through which mitogenic effects are mediated are unknown. The present study sought to determine the nature of fibrin (ogen) receptors expressed on human fibroblasts which interact with the fibrinogen Bβ chain. Receptor complexes were isolated from 125I-surface-labeled fibroblasts and purified on a fibrinogen Bβ chain affinity column. Subsequent high performance liquid chromatography and SDS-polyacrylamide gel electrophoresis analysis indicated fibrinogen Bβ chain bound specifically to a 60-kDa surface protein. Sequence analysis of the amino terminus of this protein indicated 100% homology to human calreticulin. Immunoprecipitation experiments employing a polyclonal anti-calreticulin antibody provided further evidence that the 60-kDa protein isolated in this study was calreticulin. Further, polyclonal antibodies to human calreticulin significantly inhibited the mitogenic activity of fibrinogen Bβ chain on human fibroblasts.

The cell surface components responsible for binding Aα and Bβ chains of fibrinogen are unknown. The present study sought to determine the nature of fibrinogen receptor(s) involved in binding and subsequently mediating the mitogenic action of partially degraded fibrin(ogen). Our approach utilized human fetal fibroblasts which were surface-labeled with 125I. Membrane proteins were extracted with a mild neutral detergent, and plasma membrane components were purified on a fibrinogen Bβ chain affinity column. Protein moieties eluted from the column were further purified by HPLC1 and visualized by SDS-PAGE. A 60-kDa surface protein which binds specifically to the Bβ chain of fibrinogen was identified. Sequence analysis of the first 13 amino acids of this protein showed 100% homology with human calreticulin. Further, polyclonal antibodies to human calreticulin significantly inhibited the mitogenic activity of the fibrinogen Bβ chain on human fetal fibroblasts.

EXPERIMENTAL PROCEDURES

Fibroblast Replication Assay—Fibroblast replication was assessed using a dye binding assay based on the uptake and subsequent release of methylene blue as described previously by Oliver et al. (5). Human fetal lung fibroblasts (HFL-1) were plated at 5 × 104 cells/well in 50 μl of serum-free DMEM in 96-well plates, either 24 h before the test solutions were added or concurrently with test solutions. A 50-μl sample of test solution in serum-free medium was serially diluted across the plate. 50 μl of serum-free medium was added to 3 columns representing media controls. Plates were incubated for 48 h at 37 °C in 10% CO2 and 100% humidity. Each assay was validated as a means of determining cell replication by direct cell counting. Counts were performed on cells stained with methylene blue prior to elution of the dye.

125I Cell Surface Labeling and Extraction of Plasma Membrane Proteins—Human fetal fibroblasts (HFL-1 and IMR-90, American Tissue Type Culture Collection) were grown to confluence in roller bottles over a period of 14 to 20 days. Serum-containing cell culture media were removed, and each bottle was rinsed 5 times with 10 ml of PBS containing 1 mM Ca2+ and 1 mM Mg2+. The cell pellet was reconstituted in 1 ml of PBS containing protease inhibitors and placed on ice. It was important in the context of the present study to ensure that only cell surface proteins were labeled with 125I. To ensure that the cells remained intact prior to radiolabeling all washes were performed in PBS, pH 7.4. Additionally, cells were observed under a light microscope prior to radiolabeling, and cell membranes were found to be intact.

Glass tubes were coated with IODOGEN, and 1 ml of cell suspension was added to each tube. 500 μCi of Na125I (ICN) was added, and the mixture was incubated on ice for 12 min with occasional mixing. Finally, cells were washed several times to remove free 125I. Plasma membrane proteins were extracted from human fibroblasts.
which were grown to confluence in roller bottles and harvested as described above. The same procedure was employed for both 125I-labeled cells and unlabeled cells. Cell pellets were resuspended in the neutral detergent octyl-β-glucopyranoside + protease inhibitors (Sigma), and membrane-bound proteins were extracted overnight at 4 °C. The resulting solution was spun at 20,000 × g, and the supernatant was used immediately in the affinity chromatography assay or stored at −70 °C.

Biotinylation of Surface Proteins—Cell surface proteins were biotinylated using an ImmunoPure Sulfo-NHS-Biotinylation Kit (Pierce).

Affinity Chromatography—Affinity chromatography was performed employing purified fibrinogen Bβ chain (2) coupled through the carboxy-terminus to an Affi-Gel 102 matrix (Bio-Rad). 10 mg of purified Bβ chain was dissolved in 2 ml of 8 M urea, mixed 1:1 with Affi-Gel 102, the pH was adjusted to between 4.7 and 5.0, and the mixture was stirred gently for several minutes. 5 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide coupling reagent was subsequently added, and the pH was readjusted to between 4.7 and 5.0. The reaction was allowed to proceed overnight at 4 °C with continuous stirring. The resulting complex was packed into a Bio-Rad column support and washed extensively with 50 ml of octyl-β-glucopyranoside + PBS, pH 7.4. Plasma membrane extract was applied to the column, and bound proteins were eluted with a series of washes: 40 mM EDTA, 2M KCl, and 8M urea. Each fraction was concentrated in an Amicon Centricon-10 and dried in a Speed Vac.

HPLC Protein Purification—Protein fractions collected by affinity chromatography were further purified on a C4 reverse phase HPLC column. Dried samples were reconstituted in trifluoroacetic acid/water, and 100 μl were injected onto the top of the column. Proteins were eluted with 20–50% acetonitrile gradient. Fractions were subsequently collected from the column in 250-μl aliquots, and several column runs were performed on each protein sample resulting in a final fraction volume of between 1 and 2 ml. Each fraction was dried and counted in a γ counter. Fractions containing counts were further purified on 12.5% SDS-PAGE gels. Finally, gels were dried and signal-visualized with autoradiography. Samples which were not labeled with 125I were purified in an identical manner to labeled samples with the exception that 12.5% SDS-PAGE gels were blotted onto nitrocellulose and stained with Coomassie Blue.

Protein Sequence Analysis—Protein sequence analysis was performed using an ABI 473A protein Sequencer. Proteins to be sequenced were separated by SDS-PAGE, transferred to a Problot polyvinylidene difluoride membrane, and visualized with Coomassie Blue staining. Bands were excised, and the amino-terminal sequence was determined using an ABI model 473A protein Sequencer equipped with a model 610 data acquisition system.

Immunoprecipitation—Immunoprecipitation experiments were performed employing a polyclonal antibody to human calreticulin (Affinity BioReagents). Protein preparations were prepared with normal rabbit serum. Supernatant was collected and incubated on ice for 1 h with a 1:100 dilution of polyclonal calreticulin antibody. 8 mg of protein A-Sepharose (Sigma) was added to the antibody-antigen complex, and the mixture was incubated on ice for an additional 30 min. Protein A-Sepharose was pelleted and washed three times in lysis buffer. 50 μl of Laemmli sample buffer was added to the pellet, the sample was heated at 100°C for 3 min, and the resulting supernatant was run on a 12.5% SDS-PAGE gel. Finally, the gel was dried and signal-visualized with autoradiography film.

Immunohistochemistry—Human fetal fibroblasts were plated onto 8-well chamber slides (Nunc) and left for 24 h to allow cells to adhere. Polyclonal anti-calreticulin antibodies and nonimmune controls were incubated with cells at 37 °C in DMEM for 20 min. Cells were subsequently fixed with 2% formal saline and signal-visualized with a fluorescein-conjugated secondary antibody.

RESULTS

Mitogenic Activity of Isolated Fibrinogen Bβ Chain on Human Lung Fibroblasts—Purified fibrinogen Bβ chain stimulated fibroblast replication in vitro (Fig. 1). Maximum stimulation of fibroblasts by isolated fibrinogen Bβ chain was about 30% above media control at a protein concentration of 1.1 × 10^−6 M. Activity returned to control levels with serial dilution. For comparison, the well-characterized mitogen thrombin elicited a maximal mitogenic response of 20–30% above media control assayed under the same conditions as isolated fibrinogen Bβ chain. Fetal calf serum (5%) elicited a mitogenic response of about 100%-150% above media control.

Isolation and Characterization of Cell Surface Proteins Which Bind to the Bβ Chain of Fibrinogen—To examine the cell surface receptors which bind fibrinogen Bβ chain, fibroblasts were surface-labeled with 125I, and surface proteins were extracted and passed over a fibrinogen Bβ chain affinity column. Proteins were collected from the affinity column with a series of washes. More than 90% of bound proteins eluted with 5 ml of 40 mM EDTA. The EDTA-eluted fraction was then further purified on a C4 reverse phase HPLC column. Each fraction collected from the column was dried and counted. The bulk of 125I-labeled proteins eluted from the C4 column in a single radioactive peak at an elution gradient of 40% acetonitrile. Fig. 2A, tracks a–e shows an autoradiograph of radiolabeled proteins contained in this fraction separated by SDS-PAGE. The radioactive peak collected from the HPLC column contained predominantly a single protein moiety with an apparent molecular mass of about 60 kDa.

To identify the nature of the 60-kDa protein, a large scale preparation of unlabeled plasma membrane proteins was prepared using the affinity procedure described above. Purification of the 60-kDa protein was confirmed by Coomassie Blue staining of the isolated protein transferred to Problot (Fig. 2B, track a). The stained 60-kDa band was cut out and sequenced using an ABI protein Sequencer. Sequence analysis of the 60-kDa protein indicated an amino-terminal sequence which read: EPAYFKFQFLDG. These experiments were performed on three separate preparations, and the same sequence was recorded on each occasion. A comparison of the sequence with sequences in the protein data base found 100% homology with human calreticulin.

Further experiments were performed to determine binding specificity of fibrinogen Bβ chain to the 60-kDa protein, identified as calreticulin. Fig. 3, track a, shows the ability of CNBr fragments of fibrinogen Bβ chain to elute calreticulin from a Bβ chain affinity column. For comparison, also shown in Fig. 3, track b, is the 60-kDa protein eluted with 40 mM EDTA.

Calreticulin has previously been reported as a calcium binding protein found predominantly on the luminal side of the endoplasmic reticulum. It was therefore important for the present study to determine that calreticulin was also found on the extracellular side of the plasma membrane. Fig. 4, track a, shows an autoradiograph of 125I-labeled calreticulin purified from cells scraped from a tissue culture dish in the presence of protease inhibitors prior to 125I labeling; a clear 60-kDa band can be observed. In contrast, Fig. 4, track b, shows proteins purified in a fashion identical with those shown in Fig. 4, track a, with the exception that cells were trypsinized from
tissue culture plates prior to $^{125}$I labeling. $^{125}$I-Labeled 60-kDa protein was not isolated from cells which were harvested with the serine protease trypsin.

Additional experiments were performed to confirm the identity of the 60-kDa protein and to further determine its presence as a cell surface protein capable of binding to fibrinogen B$\beta$ chain. Fig. 5, track a, shows an autoradiograph of $^{125}$I-surface-labeled proteins immunoprecipitated with a polyclonal anti-calreticulin antibody. Fig. 5, track b, shows a repetition of the immunoprecipitation experiment in Fig. 5, track a, with the exception that cells were trypsinized prior to radiolabeling. The presence of a clear 60-kDa band (visualized in Fig. 5, track a) confirms that the protein isolated from plasma membranes and purified on a fibrinogen B$\beta$ chain affinity column is a cell surface form of calreticulin. Further, the low intensity of the 60-kDa band visualized in Fig. 5, track b, provides additional evidence that the 60-kDa protein is present on the cell surface and is thus sensitive to trypsin degradation. Tracks c and d of Fig. 5 show IgG controls for both scraped and trypsinized cells, respectively.

In addition to surface-labeling cells with $^{125}$I, cells were also surface-biotinylated. Fig. 6, track b, shows biotinylated surface calreticulin immunoprecipitated with anti-calreticulin antibodies. Finally, calreticulin was visualized by immunostaining of the cell surface with polyclonal anti-calreticulin antibodies. Fig. 7A shows human fetal fibroblasts after incubation with anti-calreticulin antibodies. In contrast, Fig. 7B shows cells incubated with nonimmune IgG. Calreticulin is clearly visualized in A, and no signal was visualized with nonimmune IgG.
controls (B). Surface biotinylation and immunostaining provided further evidence, using two alternative surface-labeling technique, that calreticulin is present on the surface of the plasma membrane.

Antibody Blocking Studies—In an attempt to determine the function of the calreticulin/fibrinogen Bβ chain interaction, immune IgG raised against human calreticulin was employed to block the mitogenic activity of the fibrinogen Bβ chain (Fig. 8). In this series of experiments, the Bβ chain elicited a mitogenic response of between 63 and 42% above control. Polyclonal calreticulin antibodies significantly reduced the mitogenic activity of the Bβ chain from 63% above media control to 33% above media control at a 1:500 dilution and from 44% above media control to 31% above media control at a dilution of 1:1000. A 1:5000 dilution of antibodies neither stimulated nor inhibited fibrinogen Bβ chain activity. IgG controls tested over a comparable range of concentrations to those employed to test the effects of anti-calreticulin antibodies on Bβ chain activity neither stimulated nor inhibited the mitogenic effect of the fibrinogen Bβ chain.

**DISCUSSION**

It is well established that partially degraded fibrinogen (a fibroblast mitogen) is a fibroblast mitogen (1), an action which is mediated in part by sites within the Bβ chains of the molecule (2). It remains to characterize receptors expressed by fibroblasts which interact with fibrinogen Bβ chain and possibly mediate its biological functions. The present study isolated a membrane-bound protein which specifically bound to the Bβ chain of fibrinogen. The purified protein displayed an apparent molecular mass of about 60 kDa as determined by SDS-PAGE analysis. Sequence analysis of the purified 60-kDa protein has shown it to have an amino-terminal sequence identical with that of calreticulin. The 60-kDa protein was further identified through immunoprecipitation experiments and immunohistochemistry employing polyclonal anti-calreticulin antibodies.

Calreticulin has been described by several independent groups as a calcium-binding protein with a molecular mass of 46 kDa (6). The apparent anomaly between the molecular size determined by SDS-PAGE analysis and molecular size determined from cDNA is thought to be a consequence of the low isoelectric point of calreticulin which may result in its slow migration through SDS-PAGE gels (7). In addition to the well characterized role of calreticulin as a major calcium storage protein of the endoplasmic reticulum (8), calreticulin also displays a number of diverse activities which have direct effects on cell function. For example, a recent study by Burns et al. (9) showed that calreticulin binds to the DNA-binding domain of the glucocorticoid receptor; an event that prevented receptor-ligand interaction. These results suggest that calreticulin may play a direct role in gene transcription by regulating receptor activity. Additionally, calreticulin binds the highly conserved KXGFKKR sequence found in the cytoplasmic domain of all α-integrin subunits (10), thus mediating cell attachment to the extracellular matrix (11). The KXGFKKR sequence found as a component structure of α-integrin subunits is similar to the sequence found in the DNA-binding domain of the glucocorticoid receptor. It has been speculated that these two binding events are coordinated and that calreticulin may mediate the transduction of signals from integrins to the nucleus (11).

Calreticulin was originally thought to be confined to the endoplasmic reticulum (ER) on account of its carboxyl-terminal KDEL sequence; a sequence known to play a role in the retention of proteins within the ER (12). It has become apparent over
the last few years that, although a large proportion of intercellular calreticulin is retained within the ER, it is also found in several other locations. For example, calreticulin is found as a component of the nuclear envelope (13), it is also associated with component proteins on the cytosolic side of the plasma membrane. Additionally, calreticulin has been identified on the cell surface of human leukocytes, platelets, and endothelial cells (14, 15). Release of cell surface calreticulin from stimulated neutrophils is thought to play a role in some autoimmune disorders (16).

Although there have been reports of extracellular calreticulin (14–17), it was considered important to determine that the calreticulin described in the present study was expressed on the extracellular side of the plasma membrane. The first question considered was as follows. Did the 125I-cell surface-labeling procedure label only surface proteins? The efficiency of cell surface labeling was verified using the protease trypsin. 125I-labeled 60-kDa protein was not eluted from a fibrinogen Bβ chain affinity column when cells had been incubated with trypsin prior to 125I labeling. This observation suggested that iodination experiments performed in the present study labeled exclusively proteins expressed on the cell surface. Additionally, 125I-labeled and biotinylated surface calreticulin was immunoprecipitated with a polyclonal antibody, and a clear 60-kDa band was observed. However, immunoprecipitation of calreticulin from cells trypsinated prior to 125I-labeling yielded only a very small quantity of 125I-labeled calreticulin.

Immunostaining of cultured fibroblasts for calreticulin provided further evidence that calreticulin was present as a surface component. In this series of experiments, antibodies were incubated with live cells to ensure that cell membranes were intact and, thus, that only surface calreticulin was labeled.

Finally, polyclonal antibodies to calreticulin inhibited the mitogenic activity of the fibrinogen Bβ chain. Further, IgG controls neither stimulated nor inhibited the mitogenic effects of the Bβ chain.

It is becoming apparent that the role of calreticulin is more than simply one of calcium storage. The observation that a form of calreticulin is expressed on the cell surface and has the ability to trigger cell replication is in its self interesting. In the light of the present findings, it is also of interest to note that calreticulin contains a sequence which has the potential for phosphorylation by a number of kinases (18). It has recently been observed that a simian homologue of human calreticulin is phosphorylated, a function which facilitates its binding to viral RNA (19). It is now important to determine whether the cell surface form of calreticulin described in this study is in fact phosphorylated or is capable of phosphorylating other protein components.

REFERENCES
1. Gray, A. J., Bishop, J. E., Reeves, J. T., Mecham, R. P., and Laurent, G. J. (1995) J. Cell Mol. Biol. 32, 684–690
2. Gray, A. J., Bishop, J. E., Reeves, J. T., and Laurent, G. J. (1993) J. Cell Sci. 104, 409–413
3. Brown, L. F., Dvorak, A., and Dvorak, H. F. (1989) Am. Rev. Respir. Dis. 140, 1104–1107
4. Gray, A. J., Reeves, J. T., Harrison, N. K., Winlove, P., and Laurent, G. J. (1990) J. Cell Sci. 96, 271–274
5. Oliver, M. H., Harrison, N. K., Bishop, J. E., Cooke, P. J., and Laurent, G. J. (1989) J. Cell Sci. 92, 513–518
6. Fliegel, L., Burns, K., MacLennan, D. H., Reithmeier, R. A. F., and Michalak, M. (1989) J. Biol. Chem. 264, 21522–21528
7. McCauliffe, D. P., Lux, F. A., Leu, T. S., Szie, L., Hanke, J., Newkirk, M. M., Bachinski, L. L., Itoh, Y., Siciliano, M. J., Reichlin, M., Sontheimer, R. D., and Capra, J. D. (1990) J. Clin. Invest. 85, 1397–1391
8. MacLennan, D. H., Yip, C. C., Iles, G. H., and Seeman, P. (1972) Cold Spring Harbor Symp. Quant. Biol. 37, 469–477
9. Burns, K., Duggan, B., Atkinson, E. A., Famuold, J. K., Nemer, M., Bleackley, R. C., and Michalak, M. (1994) Nature 367, 476–480
10. Rglani, M. V., Finlay, B. B., Gray, V., and Dedhar, S. (1991) Biochemistry 30, 9859–9866
11. Leung-Hagesteijn, C. Y., Milankov, K., Michalak, M., Wilkins, J., and Dedhar, S. (1994) J. Cell Sci. 107, 589–600
12. Pelham, H. R. B. (1989) Annu. Rev. Cell Biol. 5, 1–23
13. Opas, M., Dzielak, E., Fliegel, L., and Michalak, M. (1991) J. Cell Biol. 149, 160–171
14. Malhotra, R., Willis, A. C., Jenuisens, J. C., Jakson, J., and Sim, R. B. (1993) Immunology 78, 341–348
15. Malhotra, R. (1993) Behring Inst. Mitt. 93, 254–261
16. Eglington, P., Lieu, T. S., Zapi, E. G., Saxty, K., Coburn, J., Zaner, K. S., Sontheimer, R. D., Capra, J. D., Ghebrehiwet, B., and Tauber, A. I. (1994) J. Immunol. 152, 405–409
17. Kuwabara, K., Pinsky, D. J., Schmidt, A. M., Benedict, C., Brett, J., Ogawa, S., Broedman, M. J., Marcus, A. J., Siciliano, M. J., Whitaker, R., Michalak, M., Wang, P., Pan, Y. C., Grunfeld, G., Patton, S., Malinski, T., Stern, D. M., and Ryan, J. (1995) J. Biol. Chem. 270, 8179–8187
18. Michalak, M., Milner, R. E., Burns, K., and Opas, M. (1992) Biochem. J. 285, 681–692
19. Singh, N. K., Atreya, C. D., and Nakhsh, H. L. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12770–12774