Characterization of the Receptor Responsible for Thrombin-induced Intracellular Calcium Responses in Osteoblast-like Cells*

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The receptor responsible for the increase in intracellular calcium concentration ([Ca2+]i) after the addition of thrombin to the human osteoblast-like cell line Saos-2 has been characterized. Thrombin caused a dose-dependent increase in [Ca2+]i; a half-maximal stimulation was observed with 3.2 ± 1.1 nM thrombin. The human platelet thrombin receptor is activated by thrombin cleavage to create a new NH2 terminus that acts as a tethered ligand, and peptides based on the tethered ligand can activate the receptor independently of thrombin. Northern analysis indicated the presence of mRNA encoding the platelet receptor in Saos-2 cells, and surface expression of the receptor was demonstrated by immunocytochemistry. A tethered ligand peptide (SFLLRNPDKYPF, single-letter amino acid code) was found to increase [Ca2+]i. The maximal response to the peptide was similar to that observed with thrombin, and a half-maximal response was observed with 22 ± 6 nM peptide. The time course of the increase in [Ca2+]i with the peptide was different than that observed with thrombin; a pronounced shoulder was observed after an initial sharp rise. The phenylalanine in the second position of the agonist peptide and the arginine in the fifth position were shown to be essential for its activity. The requirement for proteolysis of the receptor for the thrombin-dependent increase in [Ca2+]i was demonstrated by two methods. Antibodies that reacted with the cleavage site of the receptor abolished the effect of thrombin on [Ca2+]i. In addition, a mutant of thrombin without catalytic activity as well as chemically inactivated thrombin failed to cause an increase in [Ca2+]i. Similar results were obtained with the rat osteoblast-like cell line UMR-106; a tethered ligand peptide based on the rat sequence induced an increase in [Ca2+]i, and antibodies to the cleavage site of the rat receptor inhibited the effect of thrombin.

Thrombin, a serine protease with an important role in the blood coagulation cascade through its cleavage of fibrinogen, has hormone-like effects on cells from many tissues, including bone. Thrombin causes bone resorption in two different assay systems in vitro (Gustafson and Lerner, 1983; Hoffmann et al., 1986). Thrombin-induced bone resorption can be inhibited by cyclooxygenase inhibitors, indicating that its effect is cell-mediated rather than due to direct proteolysis of bone matrix (Gustafson and Lerner, 1983). Osteoblast-like cell lines have been shown to proliferate in response to thrombin (Tatakis et al., 1989; Babich et al., 1990). In addition, thrombin elicits increases in prostaglandin synthesis, [Ca2+]i, and phosphoinositide metabolism in these cells (Partridge et al., 1985; Tatakis et al., 1989; Babich et al., 1990). Thus, the bone resorption-stimulating effect of thrombin is probably mediated by an osteoblast-derived factor that stimulates osteoclast activity.

The thrombin receptor responsible for platelet aggregation and secretion has recently been shown to be a member of the G-protein-coupled receptor family (Vu et al., 1991). In addition to the seven transmembrane helical domains common to all members of the family, the thrombin receptor contains an amino-terminal extension of about 70 residues, and thrombin activates the receptor by cleavage within this region. The newly created NH2 terminus of the receptor then acts as a tethered ligand. Peptides based on the sequence of a tethered ligand are able to activate the receptor in the absence of thrombin (Vu et al., 1991). Thrombin receptors homologous to the platelet receptor have also been identified on fibroblasts (Rasmussen et al., 1991; Suidan et al., 1992), endothelial cells (Vu et al., 1991), smooth muscle cells (Zhong et al., 1992), and neuronal cells (Suidan et al., 1992). There is evidence, however, that another receptor for thrombin may exist (Bar Shavit et al., 1983) and that the agonist peptides may not be able to reproduce fully the effects of thrombin in certain cell types (Vouret-Craviari et al., 1992).

The present study was undertaken to determine whether osteoblasts possess the platelet-type thrombin receptor and whether this receptor mediates physiological responses in osteoblasts. The two osteosarcoma-derived cell lines used, Saos-2 (human) and UMR-106 (rat), are well characterized osteoblast-like cells (Rodan et al., 1987; Partridge et al., 1983) that are known to exhibit an increase in [Ca2+]i, in response to thrombin (Tatakis et al., 1989; Babich et al., 1990).

EXPERIMENTAL PROCEDURES

Materials—Human α-thrombin was prepared as described by Stone and Hofstengen (1986) and was fully active. A recombinant mutant of thrombin, in which the active site serine had been mutated to an alanine (Ser-195 → Ala), was a gift from Dr. B. F. Le Bonnec (Department of Haematology, University of Cambridge). Thrombin inactivated with D-Phe-Pro-Arg-CH2Cl was prepared as described previously (Stone et al., 1997) and displayed less than 0.1% the

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activity of thrombin. Briefly, human α-thrombin was treated with
Phe-Pro-Arg-CH₂Cl until no residual activity was observed, and then
the sample was dialyzed to remove the chloromethyl ketone. Tissue
culture media and media supplements were obtained from Life Tech-
nologies, Inc. (Uxbridge, United Kingdom) and Flow (Rickman-
sworth, UK). Fura-2 acetoxymethyl ester (Fura-2/AM) and di-2-1-
ethylrhodamine isothiocyanate isomer R-conjugated swine anti-rabbit immunoglob-
ulins from DAKO (Copenhagen, Denmark). All other chemicals were
from Sigma. Activated CH-Sepharose and protein A-Sepharose were
obtained from Pharmacia LKB Biotechnology Inc. (Milton Keyens,
UK). Polyclonal Antibodies—Rabbits were immunized with the human
thrombin receptor agonist peptide SFLLRNPNKDYEFP (SFLL) or
the rat thrombin receptor agonist peptide SFLLRNPSENTFELVLP (SFFL)
conjugated by 1-(3-dimethylaminopropyl)-3-ethyl carbodi-
imide hydrochloride to BSA (Doolittle, 1986). Polyclonal antibodies were
isolated by affinity chromatography on columns of the appropriate
peptide coupled to activated CH-Sepharose. Antibodies to the carrier
protein were removed by passing the polyclonal antiserum through a
nitrocellulose filter as previously described (Suidan et al., 1992). The membrane
was prehybridized in 0.25 M sodium phosphate buffer, pH
7.2, containing 0.1% SDS, 10 mM EDTA, and 1% bovine serum albumin
(BSA). Polyclonal antibodies were
removed by a 1.3-kilobase fragment of the human thrombin
receptor cDNA (Suidan et al., 1992) was labeled using the random
primer method with [α-32P]dCTP (Feinberg and Vogelstein, 1983)
and transcribed to a specific activity of about 108 cpm/μg, and the filter was hybridized
with this probe (106 cpm/ml) in the prehybridization buffer at 65 °C.
Following hybridization, the blot was washed at 65 °C in 0.2 × SSC
and 0.1% SDS. After washing, the blot was exposed to a Kodak XAR5
film with two intensifying screens for 24 h at −70 °C.

Cell Culture—The osteoblastic osteosarcoma cell lines Saos-2 (hu-
mant) and UMR-106 (rat) were grown in a humidified atmosphere
with 5% CO2 at 37 °C in McCoy's 5A medium and Dulbecco's modified
Eagle's medium: Ham's F-12 medium (1:1), respectively. Growth media
were supplemented with 10% fetal calf serum, penicillin (100
units/ml), and streptomycin sulfate (100 μg/ml).

Northern Analysis—Poly(A)+ RNA was isolated from cell lines,
fractionated in formaldehyde, 1% agarose gels, and transferred to
a nitrocellulose filter as previously described (Suidan et al., 1992). The membrane
was prehybridized in 0.25 M sodium phosphate buffer, pH
7.2, containing 7% SDS, 10 mM EDTA, and 1% bovine serum albumin
(BSA) at 65 °C. A 1.3-kilobase fragment of the human thrombin
receptor cDNA (Suidan et al., 1992) was labeled using the random
primer method with [α-32P]dCTP (Feinberg and Vogelstein, 1983)
and transcribed to a specific activity of about 108 cpm/μg, and the filter was hybridized
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and 0.1% SDS. After washing, the blot was exposed to a Kodak XAR5
film with two intensifying screens for 24 h at −70 °C.

Intracellular Ca2+ Measurement—Cells were removed from culture dishes by mild trypsinization and prepared for [Ca2+]i measurement
essentially according to published methods (Bootman et al., 1992). Briefly, cells were washed twice in Hanks' balanced salt solution
and resuspended at 5 × 10⁴ cells/ml in an extracellular medium containing
121 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl2, 1.8 mM CaCl2, 6 mM
NaHCO3, 5.5 mM glucose, and 25 mM HEPES, pH 7.3. In all subse-
quent steps the cells were protected from light. Cells were loaded with
1 μM Fura-2/AM by gentle shaking for 30 min at room temperature.
After centrifugation, cells were resuspended in an extracellular
medium and shaken for 30 min to allow hydrolysis of the intracellular
Fura-2/AM. Cells were then centrifuged and resuspended in an
extracellular medium at 2.5 × 10⁵ cells/ml for fluorescence measure-
ments in stirred quartz cuvettes.

The abbreviation used is: BSA, bovine serum albumin.

RESULTS

Northern Analysis of Saos-2 Cells—To investigate the poten-
tial role of a platelet-type thrombin receptor in the recep-
tor's role in osteoblasts, Northern analysis was
performed using a cDNA for this receptor as the probe (Fig. 1).
Samples of mRNA isolated from K562 E11, a leukemic
bone cell line showing megakaryocytic/erythroid characteristics, and
HL60, a myeloid leukemic cell line, were used as positive
and negative controls, respectively (Howells et al., 1993). A high
level of expression of thrombin receptor mRNA was
detected in Saos-2 cells. There was no detectable hybridiza-
tion of mRNA isolated from the rat cell line UMR-106 with
the human thrombin receptor probe under these high strin-
gency conditions.

Reactivity of SFLL Antibodies with Saos-2 Cells—SFLL
antibodies (4.2 μg/ml) reacted strongly with Saos-2 cells when
tested by indirect immunofluorescence (Fig. 2, A and B).
Uniform cell surface staining was observed on virtually all cells. Cells incubated with control antibodies remained un-
stained (Fig. 2, C and D). Absorption controls were performed to
assess the specificity of SFLL antibody binding. Preab-
soption of SFLL antibodies with an excess of SFLL signifi-
antly decreased staining.

The abbreviations used are: BSA, bovine serum albumin.

Fig. 1. Northern analysis. Poly(A)+ RNA was isolated from the
dicated cell lines and analyzed by Northern blot for thrombin
receptor mRNA. Approximately 3 μg of RNA was loaded in each lane. Analysis of the abundance of glyceraldehyde-3-phosphate dehydro-
genase mRNA demonstrated that equivalent amounts of mRNA had
been loaded per lane. The positions of RNA size standards are shown on
the left.
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**Fig. 2. Immunocytochemistry of Saos-2 cells.** Fluorescence microscopy of Saos-2 cells stained with SFLL antibodies (A) or control antibodies (C) is shown. Exposure time was 20 s for each fluorescence micrograph. B and D, phase contrast microscopy of the same fields shown in A and C, respectively. Bar = 50 μm.

significantly decreased cell staining (data not shown). Permeabilization of cells prior to staining markedly changed staining patterns (data not shown), confirming that SFLL antibodies bind to a cell surface antigen under nonpermeabilized conditions.

**Effect of Thrombin on Saos-2 and UMR-106 Cells**—Treatment of Saos-2 cell populations with thrombin caused a sharp, transient increase in [Ca^{2+}], followed by a gradual (within 160 s) decline to baseline (Fig. 3B(i)). The level of the transient increase in [Ca^{2+}], observed was dependent on the concentration of thrombin as shown in Fig. 3A. Analysis of these data according to the Hill equation yielded estimates of 162 ± 38 nM for the maximal [Ca^{2+}], rise and 3.2 ± 1.1 nM for the concentration of thrombin yielding the half-maximal effect (EC_{50}). The Hill coefficient (n) was 0.7 ± 0.2. As shown in Fig. 3B(iii), treatment of Saos-2 cells for 30 min with SFLL antibodies (17 μg/ml) completely abolished the [Ca^{2+}], rise in response to a maximal dose of thrombin (122 nM). The same concentration of control antibodies under the same conditions had no significant effect on the cells' response to thrombin (Fig. 3B(ii)). Treatment with either of the antibodies did not affect the response of Saos-2 cells to a maximal dose (200 nM) of the unrelated agonist bradykinin (data not shown). The effect of the antibodies did not appear to be due to antibody-induced internalization of the receptor. Preincubation with SFLL antibodies (17 μg/ml) for 30 min did not result in significant internalization of the receptor, as determined by indirect immunofluorescence. No differences in the pattern or intensity of cell surface staining of the thrombin receptor were observed between untreated cells and cells treated with SFLL antibodies prior to washing and fixing the cells for immunocytotoxicological detection of the receptor (data not shown). These results suggest that the response of Saos-2 cells to thrombin depends on the cleavage of a receptor homologous to the platelet receptor. The requirement of a cleavage event for the [Ca^{2+}], rise in response to thrombin was confirmed by the use of a thrombin mutant (Ser-195 → Ala) without proteolytic activity due to the mutation of the catalytic serine (Ser195). As shown in Fig. 3B(iv), there was no increase in [Ca^{2+}], in response to 122 nM Ser-195 → Ala. Addition of the agonist peptide SFLLRNPNDKYEPF (50 μM, see below) produced an increase in [Ca^{2+}], illustrating that the cells possessed active thrombin receptors (Vu et al., 1991). Similar results were also obtained when the cells were treated with thrombin inactivated with d-Phe-Pro-Arg-CH₂Cl; no increase in [Ca^{2+}], was observed with 24 nM d-Phe-Pro-Arg-CH₂-thrombin, but an increase was observed when the agonist peptide was added directly afterward (data not shown).

UMR-106 cells also exhibited an increase in [Ca^{2+}], in response to thrombin, followed by a more rapid (within 30 s) decline to baseline (Fig. 3C). Similarly, the increase of [Ca^{2+}], in response to thrombin was specifically inhibited by treatment of UMR-106 cells with SFLL antibodies (data not shown).

The magnitude of the thrombin-induced increases in [Ca^{2+}], with Saos-2 and UMR-106 cells was not affected by the absence of extracellular Ca^{2+}; reduction of free [Ca^{2+}] to less than 1 μM by the addition of 2 mM EGTA to the extracellular media affected neither [Ca^{2+}], peak height nor shape in response to thrombin (data not shown). Thus, the Ca^{2+} response to thrombin reflected Ca^{2+} mobilization from intra-
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FIG. 3. Effect of thrombin on Saos-2 and UMR-106 cells. A, concentration-response curve for thrombin-stimulated [Ca\(^{2+}\)]\(_i\) elevation in populations of Fura-2-loaded Saos-2 cells (mean ± S.D., 2 ≤ n ≤ 4). The values represent peak Ca\(^{2+}\) elevation above the prestimulated level. B, thrombin-stimulated [Ca\(^{2+}\)]\(_i\) elevation in populations of Saos-2 cells loaded with Fura-2. The following reagents were added: (i) 122 nM thrombin alone; (ii) 30-min preincubation with control antibodies followed by 122 nM thrombin; (iii) 30-min preincubation with SFLL antibodies followed by 122 nM thrombin; and, (iv) 122 nM Ser-195 → Ala thrombin (first arrow) followed by the agonist peptide SFLL (50 μM, second arrow). C, thrombin (122 nM)-stimulated [Ca\(^{2+}\)]\(_i\) elevation in populations of Fura-2-loaded UMR-106 cells. The arrows in B and C denote the point of addition of agonists to the cells.

cellular stores and did not require Ca\(^{2+}\) entry from the extracellular space.

Effect of Agonist Peptides on Saos-2 and UMR-106 Cells—
In order to investigate whether the thrombin-dependent transient increase in [Ca\(^{2+}\)]\(_i\) is due to activation of a receptor homologous to that found in platelets, the response of Saos-2 cells to SFLL was tested. This peptide corresponds to the tethered ligand generated by thrombin cleavage (Vu et al., 1991). Treatment of populations of Saos-2 cells with this peptide resulted in a peak in [Ca\(^{2+}\)]\(_i\) followed by a gradual decline to baseline (Fig. 4B). The shape of the [Ca\(^{2+}\)]\(_i\) response to SFLL was different than that observed with thrombin (compare Figs. 3B(i) and 4B). The initial rise in [Ca\(^{2+}\)]\(_i\) was similar with both agonists, but the return to baseline exhibited a highly reproducible shoulder with SFLL (Fig. 4B). For the platelet thrombin receptor, the peptide in which the sequence of the first two residues is reversed (FSLLRNPDKYEFP) was inactive (Vu et al., 1991). With Saos-2 cells, this control peptide FSLLRNPDKYEFP (100 μM) caused no detectable rise in [Ca\(^{2+}\)]\(_i\) (data not shown). The level of the increase in [Ca\(^{2+}\)]\(_i\) was dependent on the concentration of SFLL as shown in Fig. 4A. These data were fitted to a Hill equation to yield an estimate of 189 ± 16 for the maximal [Ca\(^{2+}\)]\(_i\) increase with an EC\(_{50}\) of 22 ± 6 μM for SFLL and a Hill coefficient of 1.3 ± 0.4.

The tethered ligand peptide of the rat thrombin receptor has the sequence SFFLRNPSENTFELVPL (SFFL; Zhong et al., 1992); this peptide SFFL (100 μM) elicited a [Ca\(^{2+}\)]\(_i\) increase in UMR-106 cells (Fig. 4C). While the peak of [Ca\(^{2+}\)]\(_i\) observed with UMR-106 cells in response to SFFL was not as sharp as that observed with thrombin (compare Figs. 3C and 4C), it did not exhibit the same pronounced shoulder as the response of Saos-2 cells to SFLL (compare Fig. 4, B and C). In the absence of extracellular Ca\(^{2+}\) (2 mM EGTA; free [Ca\(^{2+}\)] < 1 μM), the shape and the magnitude of the peaks in [Ca\(^{2+}\)]\(_i\) in Saos-2 and UMR-106 cells in response to the appropriate agonist peptide did not significantly change (data not shown).

FIG. 4. Effect of agonist peptides on Saos-2 cells and UMR-106 cells. A, concentration-response curve for SFLL-stimulated [Ca\(^{2+}\)]\(_i\) elevation in populations of Fura-2-loaded Saos-2 cells (mean ± S.D., 2 ≤ n ≤ 4). The values represent peak Ca\(^{2+}\) elevation above the prestimulated level. B, SFLL-stimulated [Ca\(^{2+}\)]\(_i\) elevation in populations of Fura-2-loaded Saos-2 cells; C, SFFL-stimulated [Ca\(^{2+}\)]\(_i\) elevation in populations of Fura-2-loaded UMR-106 cells. The arrows denote the point of addition of 100 μM peptide to cells.
The relative importance of residues in the tethered ligand peptide for its agonist activity with Saos-2 cells was assessed by sequentially replacing each of the first five NH$_2$-terminal residues by alanine. The results are shown in Fig. 5. The response (peak increase in [Ca$^{2+}$]) of Saos-2 cells to the alanine-substituted peptides (100 prompt) is expressed as a percentage of that observed with 100 uM SFLL. As shown in Fig. 5, the phenylalanine and arginine in positions 2 and 5, respectively, were essential for activity. Replacing serine in position 1 and leucines in positions 3 and 4 with alanine rendered the peptides approximately 50% active.

**DISCUSSION**

The results presented in this paper demonstrate that the increase in [Ca$^{2+}$] is due to the activation of a thrombin receptor similar to that found in platelets (Vu et al., 1991). Northern analysis (Fig. 1) indicated that the osteoblastic cell line Saos-2 transcribes mRNA encoding the platelet-type thrombin receptor. The absence of strong hybridization of the human probe with mRNA isolated from UMR-106 cells was probably due to the high stringency conditions used; the human and rat receptor cDNA sequences display only 61.5% homology. Expression of the thrombin receptor on the cell surface of Saos-2 cells was confirmed by indirect immunofluorescence (Fig. 2, A-D). Paraformaldehyde-fixed Saos-2 cells exhibited uniform cell surface staining with SFLL antibodies, while those incubated with control antibodies remained virtually unstained. Preincubating SFLL antibodies with its antigen greatly diminished staining, further demonstrating that staining was antigen-specific.

The involvement of the platelet-type thrombin receptor in the increase [Ca$^{2+}$], caused by thrombin was established by a number of different methods. The mechanism of activation of the platelet receptor involves cleavage by thrombin within the extracellular domain. The newly created NH$_2$ terminus of the receptor then acts as a tethered ligand, and peptides with the sequence of the tethered ligand can activate the receptor (Vu et al., 1991). Saos-2 cells were shown to respond to the same agonist peptide suggesting the presence of a receptor activated by the same mechanism. A control peptide in which the sequence of the first 2 residues is inverted failed to activate the platelet receptor (Vu et al., 1991) and did not increase [Ca$^{2+}$]. Antibodies that were specific for the agonist peptide sequence, which occurs immediately carboxyl-terminally to the cleavage site, were able to block completely the increase in [Ca$^{2+}$] caused by thrombin. The same antibodies had previously been shown to inhibit thrombin-dependent platelet aggregation (Howells et al., 1993). The antibodies would prevent the activation of the receptor by binding to the cleavage site and preventing proteolysis of the receptor by thrombin. The proteolytic activity of thrombin was also shown to be essential for the increase in [Ca$^{2+}$], by using the inactive thrombin mutant Ser-195 $\rightarrow$ Ala. This mutant possesses all the binding sites of thrombin but cannot catalyze the hydrolysis of peptide bonds. Coughlin and co-workers have previously shown that the Ser-195 $\rightarrow$ Ala mutant cannot activate the platelet thrombin receptor (Vu et al., 1991; Hung et al., 1992). This mutant was unable to cause the increase in [Ca$^{2+}$], seen with thrombin in Saos-2 cells (Fig. 3B(ii)). In addition, the chemically inactivated d-Phe-Pro-Arg-Ch$_2$-thrombin also failed to elicit an increase in [Ca$^{2+}$], (data not shown). Taken together the results obtained indicate an essential role for the platelet-type thrombin receptor in the Ca$^{2+}$ response of Saos-2 cells to thrombin.

The results obtained with UMR-106 cells also strongly suggest the participation of a similar receptor in the Ca$^{2+}$ response observed upon stimulation of these cells with thrombin. An increase in [Ca$^{2+}$], in UMR-106 cells was observed with the rodent tethered ligand peptide (Fig. 4C), and antibodies that bind to the cleavage site of the rat receptor inhibited the thrombin-dependent increase in [Ca$^{2+}$], (data not shown). Thus, the data obtained for Saos-2 and UMR-106 cells demonstrate that the thrombin-dependent increase in [Ca$^{2+}$], previously described (Babich et al., 1990, 1991; Tatakis et al., 1989, 1991; Ofori-Darko and Tashjian, 1993) is due to proteolytic activation of a platelet-like thrombin receptor. The proteolytic activity of thrombin is also required for its stimulation of bone resorption (Stern et al., 1990). Consequently, it seems likely that the bone resorption-stimulating effect of thrombin is mediated through its action on osteoblasts.

The requirement for thrombin's proteolytic activity for the increase in [Ca$^{2+}$], observed in Saos-2 cells (see Fig. 3B) is at variance with the data of Ofori-Darko and Tashjian (1993), which suggest that this proteolytic activity is not required. Working with Saos-2 cells, Ofori-Darko and Tashjian (1993) demonstrated that incubation of thrombin with the reversible inhibitor t-butoxycarbonyl-d-Phe-l-Pro-dL-Lys-cF$_3$ did not affect the ability of the enzyme to cause an increase in [Ca$^{2+}$], in nigerin-acidified cells. In contrast, incubation of thrombin with this inhibitor abolished its ability to increase the cytosolic pH of the cells. These results are clearly at variance with those of the present paper. Inactivation of thrombin with the irreversible inhibitor d-Phe-Pro-Arg-Ch$_2$Cl resulted in a complete loss of its ability to cause an increase in [Ca$^{2+}$]. Moreover, the inactive mutant of thrombin (Ser-195 $\rightarrow$ Ala) also failed to elicit a Ca$^{2+}$ response with Saos-2 cells (Fig. 3B(iv)). The data of Tatakis et al. (1991) also indicate that inactivation of thrombin leads to a loss of its ability to stimulate an increase in [Ca$^{2+}$], in Saos-2 cells. The differences between the present study and that of Ofori-Darko and Tashjian (1993) could be due to the fact that Saos-2 cells in the latter study were acidified by treatment with nigericin. Alternatively, a clonal variation in the Saos-2 cells could be responsible for the differences.

Northern analysis (Fig. 1) and immunocytochemistry (Fig. 2) suggest that the thrombin receptor expressed by Saos-2 cells is highly homologous to, if not identical to, the one expressed by platelets (Vu et al., 1991). The structural requirements for the activity of agonist peptides (Fig. 5) are also very similar to those observed for the platelet receptor. Substitu-
tion of residues in the agonist peptide for alanine indicated that the phenylalanine in position 2 and the arginine in position 5 were essential for activity. Similar results were obtained when studying the ability of agonist peptides to cause platelet aggregation (Scarborough et al., 1992; Vassallo et al., 1992; Chao et al., 1992). Small differences were, however, observed; replacement of the leucine in position 4 was more detrimental to the platelet aggregation activity of the peptide. Nevertheless, the binding site for the tethered ligand in the receptor expressed in Saos-2 cells appears to be very similar to that found in the platelet receptor, which has been proposed to contain a hydrophobic site for the aromatic side chain of Phe-2' and groups capable of forming electrostatic interactions with Arg-5' (Chao et al., 1992).

While the agonist peptide SFLL caused a time-dependent increase in [Ca^{2+}], in UMR-106 cells similar to that caused by thrombin (Figs. 3C and 4C), the responses of Saos-2 cells to SFLL and thrombin displayed marked differences (Figs. 3B and 4B). In Saos-2 cells, the rapid peak in [Ca^{2+}], in response to SFLL was followed by a shoulder (Fig. 4B). As the magnitude and time course of this response remained unchanged after removal of extracellular Ca^{2+}, the shoulder component of the response of Saos-2 cells to SFLL does not represent a Ca^{2+} entry phase. A possible explanation is that SFLL is less efficient at activating thrombin receptors because it lacks favorable constraints possessed by its tethered ligand counterpart generated after thrombin cleavage. However, the observed rapid and sharp rise in [Ca^{2+}], of an equivalent magnitude to that observed with thrombin (Figs. 3A, 3B, 4A, and 4B) does not lend support to this theory. The possibility of subsets of cells differing in thrombin receptor density and thus agonist responsiveness seems unlikely, as the same degree of “shouldering” was not observed after stimulation with thrombin. Furthermore, virtually all Saos-2 cells possess uniform cell surface staining with SFLL antibodies (Fig. 2). It seems likely that the observed differences between thrombin and SFLL represent differences in the way the receptor is desensitized after stimulation by these two agonists, and we are currently examining this possibility.

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