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Filamentous Actin Disruption and Diminished Inositol
Phosphate Response in Gingival Fibroblasts Caused by
Treponema denticola

by

Po Fong Yang

A thesis submitted in conformity with the requirements
for the Degree of Master of Science
Graduate Department of Dentistry
University of Toronto

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Filamentous actin disruption and diminished inositol phosphate response in gingival fibroblasts caused by *Treponema denticola*. Master of Science. 1998. Po Fong Yang. Faculty of Dentistry, Department of Microbiology, University of Toronto.

Abstract

Treponema denticola causes actin rearrangement in human gingival fibroblasts (HGF). I hypothesized that there was a treponeme-induced disturbance in the polyphosphoinositol signal transduction pathway in HGF. The purpose of this investigation was to determine the effect of *T. denticola* on inositol phosphates (IPs) in relation to stress fiber disruption in HGF. Stress fibers were stained with rhodamine-phalloidin. Exposure to *T. denticola* caused an increase in the number of HGF showing altered stress fiber patterns by 60 min and a corresponding reduction in the total fluorescence. *T. denticola* outer membrane (OM) extract caused a similar stress fiber disruption. Challenge with different strains and species of treponemes, and the OM extract resulted in a diminished accumulation of IPs within 60 min relative to controls. Pretreatment with proteinase inhibitors did not reverse the stress fiber disruption and the diminished IP response. Therefore, *T. denticola* expresses cytopathogenic activities, which are distinct from proteolytic activities. Such cytopathogenic activities lead to diminish IPs generation and are associated with cytoskeletal disruption in infected fibroblasts.

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List of Abbreviations

ADP	adenosine diphosphate
α MEM	α -minimal essential medium
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
$[Ca^{+2}]_i$	intracellular calcium concentration
CIM	CO ₂ -independent medium
CLSM	confocal laser scanning microscope
DAG	diacylglycerol
ECM	extracellular matrix
EPEC	enteropathogenic <i>Escherichia coli</i>
FBS	fetal bovine serum
FGF	fibroblast growth factor
GAPs	GTPase activating proteins
GDP	guanine diphosphate
GNRPs	guanine nucleotide releasing proteins
GTP	guanine triphosphate
HGF	human gingival fibroblasts
IP ₃	inositol-1,4,5-trisphosphate
MAP-kinase	mitogen-activated protein kinase
MglA	glucose-galactose-binding lipoprotein A
MglB	glucose-galactose-binding lipoprotein B
Msp	major outer sheath protein
OM	outer membrane
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PIP	phosphatidyl-inositol phosphate
PIP ₂	phosphatidylinositol-4,5-bisphosphate
PKC	protein kinase C
PLC	phospholipase C
PLD	phospholipase D
PROS	pathogen-related oral spirochetes
Tir	translocated intimin receptor
TKAR	tyrosine-kinase-associated receptor
YopE	<i>Yersinia</i> outer protein E

Review of the Literature

A. Structure and classification of spirochetes

Spirochetes, many of which are difficult or impossible to culture in vitro, are motile spiral rods. All spirochetes are classified into the kingdom *Procaryotae* and the division Bacteria. They are further subclassified into the order *Spirochaetales* and the families of *Spirochaetaceae* and *Leptospiraceae* (Penn, 1992). There are thirteen genera: *Borrelia*, *Clevelandina*, *Cristispira*, *Diplocalyx*, *Hollandina*, *Leptonema*, *Leptospira*, *Mobilifilum*, *Pillotina*, *Serpulina*, *Spirochaeta*, *Treponema*, and *Spirosymplokos* (Margulis et al., 1993), which are determined physiologically and morphologically, and correlated with 16S rRNA sequences (Paster et al., 1991). Spirochetes consist of an outer membrane sheath, which surrounds a helically shaped protoplasmic cylinder enclosing the cytoplasm. Within the periplasmic space between the outer membrane and cytoplasmic membrane, spirochetes have periplasmic flagella that function to provide motility. Periplasmic flagella insert subterminally at each end of the cell into basal anchorage structures, and they wind around the protoplasmic cylinder to interdigitate in the middle of the cell. This gives rise to the expression $n:2n:n$, where n is the number of flagella at each terminus, and $2n$ is the approximate number overlapping in the midcell (Loesche, 1988; Charon et al., 1992).

Treponemes are generally regarded as obligately anaerobic organisms, but although *T. denticola* generally grows better under anaerobiosis, it has proved to be facultatively anaerobic by strict definition (Fiehn, 1989; Syed et al., 1993). Dark-field microscopy is most commonly used for the detection of spirochetes. Based on dark-field microscopy, three morphotypes, small-, medium-, and large-size spirochetes, have been established (Listgarten and Hellden, 1978). *T. denticola* is a small- to medium-size spirochete (Salvador et al., 1987) with a mostly 2-4-2 arrangement of periplasmic flagella. Similarly *T. socranskii* is a small-size spirochete with a 1-2-1 arrangement. In contrast, *T. vincentii* is a medium-size spirochete with a 5-10-5 arrangement. The nomenclature *Treponema denticola* has been used for several decades. The type strain ATCC 35405, serovar "a" was first described in detail by Cheng et al. (1985), but its name was validated only recently by publication in the International Journal of Systematic Bacteriology (Chan et al., 1993). *Treponema denticola* is the species of oral spirochete that is isolated and cultivated most frequently from subgingival plaque (Cheng et al., 1985; Salvador et al., 1987).

The increased frequency, proportion and types of treponemes found in various types of periodontal diseases are well documented. Whether causal or not, treponemes are valuable indicator organisms for monitoring the status of periodontal diseases. Their presence in health-associated plaque is

associated with increased susceptibility to periodontitis (Riviere et al., 1997). As long as healthy gingival conditions exist, treponemes are either present in low numbers or are nondetectable. In gingivitis, treponemes usually constitute 2-10% of the microbiota in subgingival plaque (Greenstein and Polson, 1985; Mikx et al., 1986). In acute necrotizing gingivitis, spirochetes may constitute up to 30% of the plaque microbiota (Loesche and Laughon, 1982), and the spirochetes regularly invade gingival tissue (Courtois et al., 1983; Listgarten and Lewis, 1967; Riviere et al. 1991b).

B. Putative virulence factors of oral spirochetes

Partly due to inadequate laboratory methods for cultivation, knowledge about putative virulence factors of oral treponemes is mostly limited to small-sized species like *T. denticola*. Small-size treponemes appear to exert their detrimental effects through: (1) invasion of tissues, (2) production of tissue-destructive enzymes, (3) formation of cytotoxic products, and (4) suppression of host-cell functions.

(1) Invasion of tissues. Using monoclonal antibodies to enumerate spirochetes in dental plaque gathered from healthy controls and patients with periodontal disease, Riviere et al. (1992) showed that pathogen-related oral spirochetes (PROS) and *T. denticola* together comprise the majority of

all spirochetes. PROS and *T. denticola* serovars "b" and "d" were most numerous in plaque obtained from patients with periodontitis. The PROS share putative pathogen-specific antigens with the known invasive pathogenic spirochete of syphilis, *Treponema pallidum*, and were monitored using a cross-reactive monoclonal antibody against a pathogen-specific 37-kDa endoflagellar sheath antigen from *T. pallidum* subsp. *pallidum*. This monoclonal antibody does not react with *T. denticola*, *T. vincentii*, or *T. socranskii* (Riviere et al., 1991a, 1992). The term "PROS" may itself be misleading, as cross-reactivity with a single epitope on *T. pallidum* does not necessarily indicate that a strain is "pathogenic" or "pathogen-related". Indeed, recent comparisons of nucleotide and amino acid sequences of cloned *T. denticola* proteins with other oral spirochetes have found high homologies in the major outer sheath protein (Msp) (Fenno et al., 1996, 1997). Also, genes coding for the glucose-galactose binding lipoproteins (MglA and MglB) in *T. denticola* ATCC 35405 and *T. pallidum* are highly homologous (Becker et al., 1994; Lépine and Ellen, 1998; *T. pallidum* Molecular Genetics Web site <http://utmmg.med.uth.tmc.edu/treponema/tpall.htm/>).

During invasion of periodontal tissues, spirochaetes are found in the epithelium, connective tissue, and even on the surface of the alveolar bone (Listgarten and Lewis, 1967; Carranza et al., 1983; Frank, 1980; Saglie et al., 1982, 1985; Riviere et al., 1991c). *T. denticola* strains bind to epithelial

cells and fibroblasts (Olsen, 1984; Weinberg and Holt, 1990; Baehni et al., 1992; Keulers et al., 1993 a, b; De Filippo et al., 1995).

(2) Production of tissue-destructive enzymes. Small-size oral spirochetes exhibit activities of mucopolysaccharidases, proteases, peptidases, and phosphatases (Norton-Hughes and Yotis, 1990; Fiehn, 1989; Ishihara and Kuramitsu, 1995; Uitto et al., 1988b). The proteolytic enzymes of *T. denticola* can be divided into endopeptidases and exopeptidases. Examples of endopeptidases that have been described, characterized and/or cloned include elastase-like enzyme(s), trypsin-like enzyme(s), chymotrypsin-like enzyme(s) and others (Ohta et al., 1986; Mikx et al., 1992; Mäkinen et al., 1992; MacDougall et al., 1991). Mäkinen et al. (1995) purified and characterized a 78-kDa oligopeptidase, formerly named "trypsin-like" enzyme, from *T. denticola* ATCC 35405. Grenier et al. (1990) localized a 95-kDa chymotrypsin-like protease, first described by Uitto et al. (1988a) on the cell surface of *T. denticola* ATCC 35405. This enzyme is capable of degrading major protein components of the basement membrane, such as type IV collagen, laminin, and fibronectin (Grenier et al., 1990), and is capable of directly damaging epithelial cells (Uitto et al., 1995). Recently, Ishihara et al. (1996) reported the isolation of a possible second chymotrypsin-like serine protease from *T. denticola* ATCC 35405. This purified enzyme consisted of three polypeptides (38-, 43-, and 72-kDa). The

gene encoding the 72-kDa chymotrypsin-like protease was isolated, sequenced, and designated *prtP*. The nature and function of the 43-kDa protein have not yet been determined. Two additional chymotrypsin-like proteases with molecular weight of 30-kDa and 67-kDa from *T. denticola* ATCC 35405 have also been reported (Que and Kuramitsu, 1990; Arakawa and Kuramitsu, 1994).

Examples of exopeptidases isolated from *T. denticola* include proline iminopeptidases (Mäkinen et al., 1996) and other aminopeptidases (Holt and Bramanti, 1991; Ohta et al., 1986; Uitto et al., 1986; Mäkinen et al., 1986; Mäkinen et al., 1995; Syed et al., 1993; Mäkinen et al., 1992). In vivo, these proteolytic enzymes may provide nutrients and protect the treponemes against the activity of host salivary and crevicular-derived immunoglobulins. At the same time, the proteases may play an important role in the invasion and destruction of periodontal tissues both by direct proteolysis and by the activation of mammalian proteases and the classical and alternative complement pathways (Schenkein and Berry, 1991). The breakdown products derived in turn serve as growth factors for the treponemes. To this date, the chymotrypsin-like protease PrtP is the only extracellular enzyme of *T. denticola* known to degrade human extracellular matrix proteins.

Siboo et al. (1989) reported the synthesis and secretion of phospholipase C by *T. denticola* and *T. vincentii*, and the subsequent purification of this enzyme (Chan et al., 1991). Also, *T. denticola* has hemagglutinating and hemolytic activities (Grenier, 1991; Mikx and Keulers, 1992). Several of the hemolysins have been purified and cloned (Chu et al., 1995; Karunakaran and Holt, 1994; Chu and Holt, 1994). Fibrinolytic and gelatinolytic activity are also described (Rosen et al., 1994; Fiehn, 1989).

(3) Formation of cytotoxic products. Both *T. denticola* and *T. vincentii* adhere in vitro to epithelial cells, fibroblasts and matrix proteins in a polar orientation (Dawson and Ellen, 1990, 1994; Olsen, 1984; Reijntjens et al., 1986; Weinberg and Holt, 1990). Darkfield microscopy observation of subgingival debris often reveals spirochetes bound by one end to other bacterial masses, to erythrocytes, and to leukocytes. A 64-kDa outer sheath protein, isolated from *T. denticola* GM-1, is important in the adherence of some *T. denticola* strains to human fibroblasts (Weinberg and Holt, 1990, 1991). Fab fragments of monospecific polyclonal antibodies raised against the 64-kDa protein inhibit the adherence of *T. denticola* GM-1 to human fibroblasts by 78%. This suggests that the 64-kDa outer sheath polypeptide functions to mediate the attachment of *T. denticola* to human gingival fibroblasts (HGF). Also, the 53-kDa major surface protein (Msp) located in the outer membrane of *T. denticola* ATCC 35405 has both pore-

forming activity and adhesin activity (Haapasalo et al., 1992; Egli et al., 1993; Mathers et al., 1996; Fenno et al., 1996). Hence this 53-kDa major surface protein may play a role in the attachment of treponemes to host proteins and, thus, be an important virulence determinant (Haapasalo et al., 1992). This protein is also a major surface antigen (Umemoto et al., 1989; Koikeguchi et al., 1994). The recently released *T. pallidum* genome has 12 repeats of sequence homologs to *T. denticola* Msp (*T. pallidum* Molecular Genetics Web site <http://utmmg.med.uth.tmc.edu/treponema/tpall.htm/>).

Under some experimental conditions, *T. denticola* and *T. vincentii* can be cytotoxic to human epithelial cells (Holt and Bramanti, 1991) and to other mammalian cells (Boehringer et al., 1986; Shenker et al., 1984). They inhibit fibroblast (Boehringer et al., 1984) and endothelial cell (Taichman et al., 1984) proliferation. Some of the cytotoxic effects may be derived from the bacterial peptidoglycan (Grenier and Uitto, 1993). Much of the cytotoxic activity is loosely bound since both culture supernates and spirochete washings cause detachment of cultured epithelial cells (Reijntjens et al., 1986). Small-size oral treponemes also produce indole, H₂S, ammonia, and low molecular weight organic acids as end products that may be cytotoxic (Yotis et al., 1991).

(4) Suppression of host-cell functions. Electron microscopy of periodontal pockets has shown that spirochetes in subgingival plaque are in direct contact with the pocket epithelium and with polymorphonuclear leukocytes (Listgarten, 1976). *T. denticola* may inhibit essential functions of polymorphonuclear cells (Boehringer et al., 1986; Hurlen et al., 1984; Lindhe and Socransky, 1979; Lingaas et al., 1983; Olsen et al., 1984; Taichman et al., 1982). It produces a low-weight molecule, which is excreted into the growth medium, designated as "oral treponemal factor". Oral treponemal factor inhibits superoxide production in polymorphonuclear leukocytes (Sela et al., 1988). Treponemes may thereby suppress both phagocytosis and the oxygen-dependent bactericidal activity of polymorphonuclear leukocytes (Sela et al., 1988; Loesche et al., 1988). Sonicates of some *T. denticola* strains also cause a dose-dependent suppression of human lymphocyte proliferative responses to both mitogen and antigen in vitro (Shenker et al., 1984). The suppression involves alterations in host DNA, RNA, and protein synthesis (Shenker et al., 1984).

Using KB epithelial cells in vitro as a model, De Filippo et al. (1995) studied the early responses to *T. denticola*. They reported a progressive reduction of F-actin in *T. denticola*-treated KB cells, the loss of cell volume regulation, and the loss of cytokeratin and desmoplakin expression. In a small subpopulation, the adhesion of *T. denticola* to epithelial cell

membranes resulted in cell detachment and cell death. Ultimately, the bacteria disrupt the integrity of epithelial cell sheets. Similar spirochete-induced cytopathic effects were reported by Uitto et al. (1995) in porcine periodontal ligament epithelial cell cultures. *T. denticola* also caused reductions in the transepithelial electrical resistance of cell monolayers. In addition, in multilayered epithelial cultures, Uitto et al. (1995) observed rapid transport of *T. denticola* chymotrypsin-like proteinases both between the cells and into newly formed intracellular vacuoles within the epithelial layers, leading to loose cell contacts, collapsed intercellular spaces and increased permeability.

The adverse effects of oral spirochetes on epithelial cells are consistent with an earlier investigation by Baehni et al. (1992), who used a HGF model. Upon exposure to *T. denticola*, fibroblast monolayers showed retraction of cellular pseudopods, membrane blebbing, microvilli formation, rounding up, and detachment from the substratum. These morphological alterations were preceded by F-actin rearrangement. In both epithelial cells and fibroblasts, oral spirochetes cause changes in actin assembly. Therefore, the mechanisms involved and factors influencing actin assembly and disassembly warrant further consideration in the next section.

C. Actin assembly and stress fibers

The structure and function of actin have been well studied and have been summarized recently by Kabsch and Vanderkerckhove (1992), Herman (1993), and Reisler (1993). Actin filament polymerization and disassembly involve exchange of subunits at the filament ends, which are controlled in vitro and in vivo by other proteins that bind actin filaments and inhibit subunit addition or loss at the ends. Actin filaments assemble via noncovalent interactions between monomeric subunits (G-actin) in vitro and in vivo.

In vitro studies have shown that actin subunits polymerize and depolymerize at the ends of the linear helical actin filament. The subunits in a filament are oriented in the same direction, generating a polarity to the filament. The filament ends are called barbed and pointed ends based on decoration of filaments with myosin fragments. The two ends have different rates and equilibrium constants for their interaction with actin subunits. Each subunit binds a molecule of ATP, which is hydrolyzed to ADP after the subunit is incorporated into the filament. This energy of hydrolysis contributes to polymerization.

In vivo, actin assembly is highly controlled in time and space. Cells

maintain a large pool of unpolymerized actin at a concentration far greater than the critical concentration for polymerization in vitro. A biochemical approach to how actin assembly is regulated in vivo has uncovered a large number of actin-binding proteins that interact with filament ends and influence polymerization in vitro. In addition, exchange of actin subunits occurs in focal adhesions as evidenced by studies of fluorescently labeled actin microinjected into cells (Kreis et al., 1982; Wang, 1984).

In fibroblasts, actin filaments exist principally in three types of structures, the cortical actin network, actin stress fibers, and cell surface protrusions including membrane ruffles and microspikes (Small, 1988). Stress fibers, composed of bundles of aligned microfilaments, emanate from focal adhesions.

Actin, myosin, tropomyosin, and α -actinin each are associated with stress fibers as determined by immunological labeling techniques (Lazarides, 1975; Lazarides and Weber, 1974; Lazarides and Burridge, 1975; Weber and Groeschel-Stewart, 1974) and in the case of actin, by specific actin-binding agents, such as fluorescent phalloxin and fluorescently labeled heavy meromyosin (Sanger, 1975a; Wulf et al., 1979). The localization of myosin, tropomyosin, and α -actinin is in a banded pattern along the stress fiber analogous to patterns seen in the sarcomeres of striated muscle (Pepe,

1975), whereas the pattern of actin distribution is usually (Lazarides and Weber, 1974) but not always (Begg et al., 1978; Gordon, 1978; Sanger, 1975b) continuous along the fibers. When fluorescently labeled actin is microinjected into living cells, a uniform distribution of fluorescence in stress fibers is observed (Kreis et al., 1979; Sanger et al., 1980). This finding differs from the banded distribution seen after microinjection of either fluorescently labeled α -actinin (Feramisco, 1979; Kreis and Birchmeier, 1980) or tropomyosin (Wehland and Weber, 1980).

Ultrastructural studies have shown that parallel arrays of actin filaments are aligned along the entire length of the stress fibers, with adjacent microfilaments often opposite in polarity to one another (Begg et al., 1978; Sanger and Sanger, 1980). Myosin-like filaments have also been detected (Goldman, 1972; Rash et al., 1972), as well as osmiophilic densities spaced along the stress fibers after osmium tetroxide treatment (Giacomelli et al., 1970; Rohlich and Olah, 1967; Spooner et al., 1971). After comparing the immunofluorescence banding patterns due to myosin and α -actinin with the distribution of the densities, it was proposed that these densities are sites of α -actinin (Gordon, 1978) and myosin (Goldman et al., 1979) localization. It has also been demonstrated that ferritin-labeled antimyosin localizes to the ultrastructural densities and, at lower concentration, between the densities, as well (Herman and Pollard, 1981).

With the above physical description of stress fiber structures in mind, emphasis will now be shifted towards factors that influence actin dynamics. The presence of a large array of actin-binding proteins (for review, see Hartwig and Kwiatkowski, 1991), which bind to actin and modulate its properties and functions, is one example of factors that influence actin dynamics. In a fibroblast cell approximately 50% of the actin is in filaments and 50% is in monomer form (Fechneimer and Zigmond, 1993). Profilin is one of the proteins that are involved in the filamentous-monomeric inter-conversion dynamics. Profilin binds to G-actin monomers, sequestering them, and thus lowering the concentration of free subunits. This shift in the steady state delays polymerization and induces depolymerization of actin filaments. Profilin may also deliver an actin monomer to the filament by the profilin-G actin complex binding to the barbed end, followed by the rapid dissociation of the profilin (Pring et al., 1992). Profilin can enhance actin polymerization by catalyzing nucleotide exchange, converting polymerization-incompetent ADP-G actin, to polymerization-competent ATP-G actin (Goldschmidt-Clermont et al., 1992). Other actin-binding proteins, such as fimbrin, α -actinin, and filamin, exhibit cross-linking activities. Fimbrin and α -actinin cross-link actin filaments into parallel arrays found in microspikes, filopodia, and stress fibers while filamin cross-links actin filaments at crosswise intersections, creating loose gels. Within the large category of actin-binding proteins, there also exists severing proteins that fragment the

cross-linked network of actin filaments. One such protein is gelsolin, which when activated by the binding of Ca^{2+} , severs an actin filament and forms a cap on the newly exposed end of the filament (McLaughlin et al., 1993).

Besides actin-binding proteins, evidence has begun to emerge that the Rho family of GTP-binding proteins is also involved in regulating the actin cytoskeleton in response to extracellular factors (Downward, 1992). When Rho is microinjected into serum-starved fibroblasts, rapid formation of actin stress fibers is observed (Ridley and Hall, 1992). The addition of serum results in a similar effect (Ridley and Hall, 1992). The component in serum responsible for this effect is lysophosphatidic acid (Ridley and Hall, 1992). Additional factors involved in actin cytoskeleton regulation that have received intense study in recent years are elements involved in the inositol phospholipid signaling pathway discussed next.

D. Inositol phospholipid pathways

Upon binding to cell surface receptors, many extracellular signals initiate secondary messenger signaling pathways whereby information is relayed through a cascade of components until the final effector system is activated. The inositol phospholipid pathway is an example of such a messenger system. Its core pathway is the hydrolysis of PIP_2 (phosphatidylinositol 4,5-bisphosphate) by activated phosphatidylinositol (PI)-specific phospholipase C (PLC) to give both diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) (Fig. 1 and 2). The formation of DAG and IP_3 is the converging point for two major pathways, one initiated by a family of G protein-linked receptors and the other by receptors linked by tyrosine kinases either directly or indirectly. These separate receptor mechanisms are coupled to energy-requiring (GTP or ATP) mechanisms (Nishizuka, 1988; Berridge, 1993).

PIP_2 is formed by sequential phosphorylation of inositides at the 1, 4 and 5 positions of the inositol ring by lipid kinases. The IP_3 released into the cytoplasm then binds to an IP_3 receptor in the endoplasmic reticulum to mobilize internally-stored calcium and to subsequently promote an influx of external calcium, thus affecting Ca^{2+} -regulated processes in the cell (Berridge and Irvine, 1989; Rana and Hokin, 1990; Henzi and MacDermott,

Figure 1. The basic structure of phosphatidyl inositol. In phosphatidyl inositol, the hydroxyl groups at C-1 and C-2 of glycerol are esterified to the carboxyl groups of two fatty acid chains. The C-3 hydroxyl group of the glycerol backbone is esterified to phosphoric acid. The phosphate group becomes esterified to the hydroxyl group of an alcohol, such as inositol. The diagram on the right is a symbolic representation of the chemical structure on the left. (modified from Alberts et al., 1994)

Figure 2. Inositol phospholipids and the hydrolysis of PIP₂. The polyphosphoinositides PI-4-phosphate and PI-4,5-bisphosphate (PIP and PIP₂ respectively) are produced by the sequential phosphorylation of phosphatidylinositol (PI). Two intracellular mediators are produced when PIP₂ is hydrolyzed: inositol-1,4,5-trisphosphate (IP₃), which diffuses through the cytosol and releases Ca²⁺ from the endoplasmic reticulum, and diacylglycerol (DAG), which remains in the membrane and helps activate the enzyme protein kinase C (PKC). (modified from Alberts et al., 1994)

1992; Ferris and Snyder, 1992). On the other hand, DAG is the physiological activator of protein kinase C (PKC). PKC can phosphorylate mitogen-activated protein (MAP)-kinase-kinase-kinase, which in turn, through a sequential phosphorylation cascade, can activate MAP-kinase. Activated MAP-kinase, which functions as a serine/threonine-specific kinase, can in turn phosphorylate a host of proteins, and has been implicated in the control of cellular growth.

Three families of PI-PLC (β , γ , δ) are known (Cockcroft and Thomas, 1992; Rhee and Choi, 1992). The PLC $_{\beta}$ subfamily is regulated by G protein coupled to receptors. PLC γ (isoform PLC γ_1 and PLC γ_2) contains a SH $_2$ domain, which directs interaction with the activated tyrosine-kinase-associated receptors (TKAR) (Meisenhelder et al., 1989; Rhee, 1991; Hasegawa-Sasaki, 1985; Morrison et al., 1990), and SH $_3$ domains, which localize PLC γ to the cytoskeleton (Bar-Sagi et al., 1993). Some examples of TKAR include receptors for epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and fibroblast growth factors. Association with the activated TKAR results in phosphorylation of Tyr on PLC γ_1 (Margolis et al., 1989; Meisenhelder et al., 1989; Kim et al., 1991; Meisenhelder et al., 1989; Burgess et al., 1990; Mohammadi et al., 1991; Rhee, 1991), leading to PLC γ_1 activation (Goldschmidt-Clermont et al., 1991; Wahl et al., 1992).

Epidermal growth factor- and platelet derived growth factor-receptor activation stimulate PI turnover in some cells (Kauffmann-Zeh et al., 1994). An insight into the control mechanism of PLC γ on the main IP pathway was provided by Margolis et al. (1990), who created overexpression of PLC γ_1 cDNA in NIH 3T3 fibroblasts. This resulted in increased PIP $_2$ hydrolysis observed after tyrosine kinase-associated receptor activation by growth factors (Wahl et al., 1992) and elevated growth-factor-induced IP $_3$ levels in vivo.

In summary, the transduction of extracellular stimuli from a broad series of ligands, including neurotransmitters, regulatory peptides, hormones, releasing factors, platelet activators, growth factors, and various biologically active molecules (such as ATP and bombesin), proceeds into the cell and triggers the IP $_3$ /DAG pathway (Castagna, 1987). This bifurcating messenger system operates throughout the life of a typical cell, and its constituent molecules have been implicated in the regulation of the actin cytoskeleton. The relationship of inositol phospholipid pathways and actin will be expanded in detail in the discussion section relative to the findings of this study.

E. Exploitation of host cytoskeletal elements and signal transduction pathways by pathogenic bacteria

Most of the leading research into the effects of bacteria on the cytoskeleton of host cells has concentrated on enteric pathogens because the highly dynamic nature of the actin cytoskeleton is often exploited by species such as *Yersinia*, *Salmonella*, *Shigella*, enteropathogenic *Escherichia coli* (EPEC) and *Listeria monocytogenes* during their entry and subsequent movement within and among mammalian host cells. These invasive bacteria appear to transduce signals across the host cell membrane to induce bacterial uptake. Entry of all of these bacteria requires rearrangement of the host cell actin cytoskeleton since uptake is impaired by inhibitors of actin polymerization, such as cytochalasin D. The interplay between invading microorganisms and host cell responses has been summarized recently in detail by Rosenshine and Finlay (1993), Finlay and Cossart (1997), and Cossart (1997).

Even though under in vitro conditions, *T. denticola* has been shown in transmission electron photomicrographs to locate in large cytoplasmic vacuoles of porcine periodontal ligament epithelial cells (Uitto et al., 1995) and in an intracellular location close to the nucleus in human gingival fibroblasts (Ellen et al., 1994a), *T. denticola* is not normally considered as

an intracellularly invasive microorganism. However a microorganism does not necessary have to be intracellularly invasive in order to affect host actin structures. YopE (*Yersinia* outer protein), which is produced by pathogenic bacteria of the genus *Yersinia*, and actin-ADP-ribosylating toxins (e. g. botulinum C2 toxin), which are produced by a number of clostridial bacteria, are two such examples. Upon contact with a target cell, extracellular *Yersiniae* increase expression of YopE cytotoxin (Pettersson et al., 1996) and translocate it through the host plasma membrane into the cytosol, resulting in disruption of the host cell actin microfilament structure (Rosqvist et al., 1991, 1994, 1995). Similarly upon entering a host cell, clostridial toxins can ADP-ribosylate host monomeric actin, which is then functionally transformed into a capping protein and binds to the barbed end of filaments to inhibit polymerization. The monomeric actin concentration is increased by depolymerization at the pointed ends of the filaments. G-actin monomers released are substrates of the toxin. As ADP-ribosylated actin loses its ability to polymerize, this finally causes breakdown of the microfilament network (for review, see Aktories and Wegner, 1989). Even though the mechanisms by which enteric bacteria and *T. denticola* affect the host actin cytoskeleton may be different, it still may be beneficial to glean insights from the enteric bacteria studies. Hence the following summary of certain enteric bacteria will highlight only changes related to the actin cytoskeleton and the accompanying signal transduction pathways upon entry.

Upon initial contact with the host cell, salmonellae and shigellae trigger dramatic rearrangements of cellular F-actin, characterized by large membrane projections (Finlay et al., 1991). The host membrane engulfs the microorganism in a large vacuole formed by fusion of membrane projections. Entry of *Salmonella* correlates closely with an increase in IP_3 production, which returns to normal levels after bacterial internalization (Ruschkowski et al., 1992). Chelation of intracellular calcium ($[Ca^{+2}]_i$), but not extracellular calcium, inhibits *S. typhimurium* uptake (Ruschkowski et al., 1992). It seems likely that the bacterium stimulates host phospholipase C leading to IP_3 production, which then mobilizes $[Ca^{2+}]_i$. Calcium fluxes may affect uptake by controlling activities of various actin-binding proteins, such as α -actinin, talin, and ezrin, which are recruited to the site of entry (Finlay et al., 1991). In contrast, diacylglycerol activation of host PKC activity is not required for *S. typhimurium* uptake into HeLa cells (Rosenshine et al., 1992b). It was recently reported that *S. typhimurium* stimulates tyrosine phosphorylation of the EGF receptor (Galan et al., 1992).

Internalization of *Shigella* appears to involve tyrosine phosphorylation of several key host proteins, including cortactin, pp125FAK, and paxillin (Dehio et al., 1995; Watarai et al., 1996). Tyrosine phosphorylation of c-Src increases during *Shigella* invasion, and this kinase is recruited to the site of entry. Cortactin, an actin-associated protein, is a

substrate for the nonreceptor tyrosine kinase c-Src. Transient overexpression of c-Src in transfected cells induces membrane ruffling and stimulates entry of normally noninvasive *Shigella* mutants, suggesting that this kinase plays a role in entry (Dehio et al., 1995). T-plastin is an actin-binding protein that appears to have a function in bacterial uptake, possibly by bundling newly formed actin filaments in membrane extensions (Adam et al., 1995). Vinculin also colocalizes to the sites of entry of *Shigella* (Tran Van Nhieu et al., 1997).

The superfamily of Ras-related small G-proteins can be divided into four subgroups: Ras-like, Rho-like, Rab-like and ADP-ribosylation factor-like (Hall, 1993). The subfamily of Rho-like proteins, which includes RhoA, B, and C, Rac1 and 2, and CDC42, also play crucial roles in the regulation of the actin cytoskeleton. Recently it was demonstrated that invasion by *Salmonella* requires CDC42 function, but not Rac or Rho (Chen et al., 1996). On the other hand, invasion by *Shigella* is Rho-dependent (Adam et al., 1996). A Rho-specific inhibitor abolished *Shigella*-induced membrane folding and impaired bacterial entry into HeLa cells (Adam et al., 1996).

Upon adherence to the epithelial cell surface, EPEC, even without invading, elicits an "attaching and effacing" phenotype (Moon et al., 1983), which is characterized by localized degeneration of the brush border

microvilli and the assembly of highly organized cytoskeletal structures in the epithelial cells just beneath the adherent bacteria (Knutton et al., 1989). This structure is composed of cytoskeletal elements, such as actin filaments, α -actinin, ezrin, talin and myosin light chain (Knutton et al., 1989; Finlay et al., 1992; Rosenshine et al., 1992a; Manjarrez-Hernandez et al., 1992).

In 1997, Kenny et al. reported that to initiate infection, EPEC first inserts its bacterial protein Tir (translocated intimin receptor) into host cell plasma membrane surfaces. Tir has three functions (Kenny et al., 1997): (a) After insertion, Tir protein becomes the receptor to which EPEC outer membrane protein intimin then adheres; (b) Following intimin binding, Tir nucleates actin to localize actin beneath adherent bacteria; and (c) Tir-intimin interaction triggers tyrosine phosphorylation of phospholipase C γ and other host proteins (Rosenhine et al., 1992a; Kenny and Finlay, 1997). This phosphorylation may subsequently trigger formation of protein aggregates, which are composed of tyrosine phosphorylated proteins and other non-phosphorylated cytoskeletal elements beneath the adherent bacterium (Rosenhine et al., 1992a). EPEC also causes increases in the concentration of IP $_3$ and [Ca $^{+2}$], in the infected epithelial cells (Baldwin et al., 1991; Dytoc et al., 1994). Furthermore, EPEC induces protein kinase C (PKC)-like activity to phosphorylate the host myosin light chain and another 27-kDa protein (Baldwin et al., 1990; Manjarrez-Hernandez et al., 1991;

Crane and Oh, 1997).

Meyer et al. (1997) made a comparative analysis of the invasion processes of enteric and periodontal pathogens into epithelial cells and showed that similar invasion strategies and mechanisms are used. The periodontal pathogens of interest here are *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*, both of which exhibit the ability to invade epithelial cells (Meyer et al., 1991, 1996; Lamont et al., 1995) and are implicated as etiologic agents in periodontitis. *A. actinomycetemcomitans* invasion of epithelial cells is a dynamic, multistep process (Meyer et al., 1996). Entry of the host cell begins when *A. actinomycetemcomitans* attaches to the epithelial cell. Attachment of *A. actinomycetemcomitans* leads to rearrangement of host cell actin from the periphery of the cell to a focal point beneath the bacterium at the point of entry (Fives-Taylor et al., 1995). *A. actinomycetemcomitans* enters the cell in a host-derived membrane-bound vacuole (Meyer et al., 1991).

In comparison, the entry of *Porphyromonas gingivalis* into host oral epithelial cells (Duncan et al., 1993; Sandros et al., 1993, 1994; Lamont et al., 1995) appears to be mediated by classic receptor-mediated endocytosis (Sandros et al., 1993, 1994, 1996). First binding to host cells and activating the epithelial cell Ca^{2+} signaling system (Izutsu et al., 1996), *P. gingivalis*

then becomes encircled by microvilli and results in internalization. The invasion is inhibited by both inhibitors of microfilaments and microtubules, suggesting that both cytoskeletal components are involved in bacterial entry (Lamont et al., 1995, Sandros et al., 1996). Sandros et al. (1996) recently reported that treatment of the epithelial cells with staurosporine (a protein kinase inhibitor) and genistein (a tyrosine-specific protein kinase inhibitor) decreased the number of invading bacteria. The identification of a 43-kDa protein acting as a substrate for tyrosine phosphorylation subsequent to the bacterial-epithelial cell interaction provided further support for the involvement of tyrosine phosphorylation in signal transduction during invasion (Sandros et al., 1996).

In summary, many enteropathogenic and putative periodontal pathogenic bacteria possess the ability to exploit the existing host signaling pathways in order to pirate the accompanying alterations in actin cytoskeleton for the purposes of bacterial entry, intracellular movement and subsequent intercellular spread. Whether *T. denticola* and related oral spirochetes, which were shown previously by us to perturb actin cytoskeleton, also exploit some of these host signaling pathways was the focus of the experiments outlined in this thesis.

Statement of the Problem

As summarized in the Review of the Literature, *Treponema denticola* exhibits strong proteolytic activities and other putative pathogenic factors. Being able to penetrate tissue, *T. denticola* cells may bring enzymes, metabolic products, and endotoxins into direct contact with target cells, thereby causing disruption of essential functions in the host cells. One of the most obvious, observable outward expressions hinting at an internal imbalance in homeostasis is the disruption of filamentous actin. There is ample evidence in the literature of studies exploring the control of F-actin assembly and disassembly by various elements involved in the polyphosphoinositol signal transduction pathway. Indeed, recent investigations on invasive bacteria demonstrate the exploitation of this inositol phosphate pathway and other linked pathways to manipulate aspects of actin polymerization to the bacteria's advantage.

In this thesis, I hypothesize that there is a disturbance in human gingival fibroblast second messenger signaling pathways induced by oral spirochetes in vitro, and that such changes normally will precede in temporal sequence the treponeme-induced changes in F-actin. Two methods of measuring F-actin changes were used and compared for quantifying actin-disruption outcome. The focus for studying signaling pathways was

restricted to the main inositol phosphate signaling pathway, as outlined earlier. The final aim was to temporally link *Treponema denticola*-induced stress fiber interruption to changes in inositol phosphate responses.

Materials and Methods

Treponemal cultures and culture conditions. Stock cultures of *T. denticola* ATCC 35405 (type strain, originally provided by W. J. Loesche, University of Michigan), ATCC 35404, e, and e' (provided by E. C. Chan, McGill University), *Treponema vincentii* ATCC 35580, and *Treponema socranskii* ATCC 35536 were maintained in a spirochete broth medium [containing, per liter, 12.5 g of brain heart infusion broth, 10.0 g of trypticase, 2.5 g of yeast extract, 0.5 g of sodium thioglycolate, 1.0 g of L-cysteine hydrochloride, 0.25 g of L-asparagine, 2.0 g of glucose, 2.0 ml of a volatile fatty acid mixture (containing, per 100 ml of 0.1 N KOH, 0.5 ml each of isobutyric acid, D,L-2-methylbutyric acid, isovaleric acid, and valeric acid), 20.0 ml of rabbit serum, and 20.0 ml of 10% sodium bicarbonate] as previously described (Dawson and Ellen, 1990). Cells were grown at 37°C and subcultured weekly in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) in an atmosphere of 80% N₂, 10% CO₂, and 10% H₂ (Canox, Toronto). For experiments in which bacterial suspensions were used to challenge human gingival fibroblasts, 3-day-old, stationary phase cultures were harvested by centrifugation at 12,000 x g for 6 min and washed twice in 0.01 M phosphate buffered saline at pH 7.2 (PBS).

***T. denticola* outer membrane (OM) preparation.** Spirochete medium was inoculated with a 3-day-old culture of *T. denticola* ATCC 35405 at a ratio of 30:1 fresh medium to inoculum and incubated at 37°C for 4 days. Bacteria were harvested by centrifugation at 12,000 x g for 15 min at 4°C and washed twice in PBS. The pellet was weighed, dispersed uniformly, and resuspended at 1 g wet weight per 10 ml PBS containing 10 mM MgCl₂. A modification of the detergent extraction method of Penn et al. (1985; E. C. Chan, personal communication) was used for the initial extraction. Triton X-100 (Surfact-Amps X-100, Pierce, Rockford, Ill.) was added to a final concentration of 0.2% (v/v). The suspension was incubated with constant mixing at 37°C for 30 min and then repeatedly centrifuged at 12,000 x g for 6 min until no visible pellet remained. The clear supernatant was dialyzed (Spectra/Por, Spectrum, Houston, Tx.; molecular weight cut-off 50 kDa, precleaned by boiling 10 min in 1 mM EDTA) against deionized H₂O at 4°C for several days until precipitates formed. The contents of the dialysis tubing were centrifuged at 25,000 x g for 45 min. The pellet was resuspended in deionized H₂O to predialysis volume and stored at -70°C. The dry weight of a lyophilized aliquot of the OM extract was determined, so the actin-perturbing activity of the extract could be compared with that of whole *T. denticola* cells on a dry weight basis.

Treponema denticola cell suspensions and the OM extract were tested

for peptidase activity using chromogenic peptides N-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-*p*-nitroanilide (SAAPNA) and N-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA; Sigma) (Ellen et al., 1994b). Whole *T. denticola* cells at the concentration used for the assays below had the equivalent SAAPNA-degrading activity of 0.2 µg/ml chymotrypsin and BAPNA-degrading activity of 0.04 µg/ml trypsin. The undiluted OM extract contained SAAPNA-degrading activity equivalent to 4.0 µg/ml chymotrypsin and no detectable BAPNA-degrading activity.

Gingival fibroblast cultures. Human gingival fibroblasts (HGF) were established from human tissue explants, as described previously (Arora et al., 1994). The cells were cultured in α minimal essential medium (α MEM) containing 400 U/ml penicillin G and 10% (v/v) fetal bovine serum (FBS, BioWhittaker, Walkersville, Maryland) and maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The fibroblasts were subcultured weekly and were used for experiments between the 14th and 25th passages.

Bacterial perturbation of F-actin in HGF. Two assays (Fig. 3a) were used to measure the degree of actin perturbation in HGF challenged by bacterial suspensions during time course experiments. The first assay documented the percentage of cells with rearranged stress fibers by

Figure 3a. Steps involved in measuring the degree of F-actin perturbation in human gingival fibroblasts in vitro by treponemes.

dichotomizing the outcome as unaltered or altered stress fiber patterns.

The second assay used confocal microscopy to measure mean fluorescence of F-actin in optical sections of the HGF.

HGF in confluent monolayers were trypsinized, washed in PBS, and adjusted to a density of 1×10^5 cells/ml in α MEM containing penicillin G and FBS. The cell suspensions were distributed at 1.0 ml/well in 24-well dishes (Corning Glass Works), each well containing one circular glass coverslip (No. 1, 12 mm in diameter, Fisher Scientific). The coverslips had been precleaned by boiling in 0.1 N HCl and washed in distilled H₂O and then sterilized. The HGF were incubated at 37°C in a 5% CO₂ atmosphere for 3 h to allow spreading. Prior to the addition of bacteria, each well was washed once with CO₂-independent medium (CIM, Gibco). The treponemes were washed, resuspended in serum-free CIM to a cell density of 2×10^9 bacteria/ml, and added to the wells containing the coverslips at 1.0 ml/well in duplicate. Control wells received CIM without bacteria. The dishes were incubated at 37°C. In a time course experiment of 140 min, HGF were fixed at 20 min intervals in 1.0 ml 3.75% (v/v) formaldehyde over night in PBS. After removing the fixative, the coverslips were washed with PBS and stained with rhodamine-phalloidin.

Phalloidin binds to the polymeric form of actin but not to its

monomers (Faulstich et al., 1988). The fluorescence of rhodamine-conjugated phalloidin is enhanced by ninefold upon binding to F-actin and can be used as a method of F-actin quantitation (Huang et al., 1992). Rhodamine-phalloidin (Molecular Probes, Eugene, Oregon), 200 units/ml, at a dilution of 30 ml/10 ml 0.1% (v/v) Nonidet P-40 (Sigma) in PBS was added at 0.5ml/well and incubated at room temperature for 30 min. The coverslips were washed twice in deionized water. HGF were examined under UV light (excitation 550nm and emission 580 nm) by conventional microscopy at 500 x magnification after mounting the coverslips onto glass slides with antifade mounting solution containing 0.1% (w/v) *p*-phenylenediamine, 10% (v/v) 0.01 M PBS, pH 7.4, 90% (v/v) glycerol, and adjusted to pH 8.0 with 0.5 M carbonate-bicarbonate buffer. At least 200 HGF on duplicate coverslips were scored dichotomously according to preset criteria as either presenting a normal or altered stress fiber pattern and the outcome was expressed as the percentage with altered stress fibers in the total. HGF scored as normal had stress fibers that were abundant in quantity, evenly and brightly fluorescent, long, mostly straight, and detected in well-spread cells. In contrast, HGF scored as altered had stress fibers that were reduced in quantity or absent entirely, fragmented and unevenly fluorescent, and found in cells with retraction of cytoplasmic processes and surface blebbing (Fig. 3b). The samples were coded to obscure their identity. Statistical significance between experimental and control groups at each time point and

a **b**

Figure 3b. Fluorescence photomicrograph of human gingival fibroblasts stained with rhodamine-phalloidin showing (a) normal pattern of stress fibers, which are abundant in quantity, evenly and brightly fluorescent, long, mostly straight, and detected in well-spread cells, and (b) altered pattern of stress fibers, which are reduced in quantity, fragmented and unevenly fluorescent, and found in cells with retraction of cytoplasmic processes and surface blebbing. Bar = 20 μm .

within-group differences between time points was determined by chi-square analysis.

A comparison of whole *T. denticola* cells and OM extract for actin-perturbing activity was made in 90-min assays using the stress fiber assay. The effect of inhibiting the proteolytic activity of the bacteria and OM extracts was also tested. They were pretreated with phenylmethylsulfonyl fluoride (PMSF) at 170 µg/ml, which had been shown previously (Baehni et al., 1992; Ellen et al., 1994b) to inhibit *T. denticola*'s fibronectin-degrading activity and detachment of HGF from the extracellular matrix.

The degree of fluorescence in optical sections of control and *T. denticola* ATCC 35405-challenged HGF was measured by confocal microscopy. The HGF were prepared as for the dichotomous stress fiber assay. The confocal laser scanning microscope (CLSM) (Leica Lasertechnik GmbH, Heidelberg, Germany) was set with an argon ion laser of 488/514 nm emission, BP568 laser filter, RSP 488/568 beam splitter, pinhole setting at 40, and offset at -30. Leica CLSM has a computer-controlled variable detection pinhole ensuring an optimum adaptation to the objective (Leica Lasertechnik GmbH, 1991). The pinhole setting was a relative value with 0 corresponding to the smallest size and 255 corresponding to the maximum size (Leica Lasertechnik GmbH, 1991). The pinhole setting was set to the

magnification size of the objective lens in use as recommended (Prinz, 1994). The offset parameter allowed adding or subtracting background to the image. Fluorescence intensity of rhodamine-phalloidin labeled F-actin was recorded in serial 1 μm optical steps in the X-Y plane at 400 X magnification (a 40X oil objective lens with a numerical apperture of 1.3) from the dorsal to the ventral (substratum) surface. Thirty cells per group per time point were examined and fluorescence intensity per section was recorded. The total fluorescence intensity in an individual cell was calculated as the sum of fluorescence in all its optical sections, and the mean fluorescence per cell was derived for the 30 cells counted per time period per experimental group. As exposure to *T. denticola* caused many of the HGF to undergo progressive changes in shape, including cellular rounding and increased height, the number of optical sections for complete coverage of each single cell was determined, and the sections were categorized into thirds by cell height. The mean fluorescence per ventral, middle, and dorsal third for the 30 cells per group was calculated. For statistical analysis, significant differences in mean fluorescence between experimental groups at each time period and within-group differences at various time periods were determined by Student's t-test.

Inositol phosphate assay. Inositol phosphates were measured by a modification of the methods of Ruschkowski et al. (1992) and Dean and

Beavan (1989) (Fig. 4). HGF in confluent monolayers were harvested by trypsinization and resuspended in α MEM with 10% FBS to a density of 2×10^5 cells/ml. The cell suspensions were dispensed (2.5 ml/60 x 15 mm petri dish, Falcon, Franklin Lakes, U. S. A.) and incubated at 37°C in a humidified atmosphere containing 5% CO₂ to allow cell spreading. After 2 h, each petri dish was washed twice with warm PBS, and 2.0 ml α MEM containing penicillin G and 1% (v/v) FBS was added. One hundred microliters of 1:10 concentrated myo-[³H]-inositol (1.0 mCi/ml, Amersham, Arlington Heights, Ill.) in 10% (v/v) ethanol was added to each dish. The dishes were incubated for at least 18 h, the culture fluids removed, and the dishes washed once with warm serum-free CIM. Two ml CIM containing 1% (v/v) FBS and 10 mM LiCl were added to each dish and incubated for 15 min at 37°C in air. The LiCl inhibits endogenous phosphatases and thereby allows the accumulation of [³H]-labeled intermediates in the IP pathway (Fig. 5).

Three-day cultures of treponemes were harvested by centrifugation, washed once in CIM, and resuspended in CIM containing 1% (v/v) FBS and 10 mM LiCl to a density of 2×10^9 bacteria/ml. Three ml were added to each petri dish of HGF in the experimental groups. The positive control dishes, in which increases in accumulated [³H]-labeled IP intermediates would be anticipated, received 3.0 ml CIM containing 15% (v/v) FBS and

Figure 4. Steps involved in measuring changes in inositol phosphates in response to bacterial challenge in vitro.

LiCl, and the background control dishes received 3.0 ml CIM containing 1% (v/v) FBS and LiCl according to Ruschkowski et al. (1992). In some experiments, ATP (100 μ M) in CIM containing 1.0% FBS, was used as the positive agonist. During a 60-min experimental time course, duplicate dishes per treatment group per 15 min interval were analyzed for inositol phosphates.

The reactions were terminated by removing the medium and washing once with ice-cold PBS. Cells in each dish were harvested into 1.0 ml ice-cold PBS and collected into screw-capped polypropylene tubes. Into each tube, 4 ml methanol : chloroform (2:1, v/v) solution was added, mixed, and allowed to react at ambient temperature for at least 30 min. Then a mixture of 0.125 ml 0.5 M EDTA, pH 8.0, 1.2 ml chloroform, and 1 ml deionized H₂O was added. The mixtures were vortexed and centrifuged at 500 x g for 8 min. The aqueous phase, which contained the water soluble inositol phosphates, was collected into a new polypropylene tube. The aqueous phase solution was passed through a 1 ml packed, deionized water-prewashed Dowex AG 1-X8 anion exchange resin column (formate form resin and Poly-Prep chromatography columns, Bio-Rad) followed by a wash of 5 ml deionized H₂O. The inositol phosphates were eluted with a 5.0 ml solution containing 0.1 M formic acid and 1.0 M ammonium formate into polypropylene tubes with screw caps.

Three hundred μ l of each sample was added to 5.0 ml scintillation fluid (Ecolume, ICN, Costa Mesa, Ca.), and the radioactivity in counts per min (cpm) was measured in the tritium channel (RackBeta Scintillation Counter, LKB-Wallac). For IP assays in which the outer membrane (OM) extract of *T. denticola* ATCC 35405 was substituted for the whole bacteria, the OM extract was serially diluted 1/4 to 1/16 in CIM containing 1.0% FBS and 3.0 ml of the suspension was added to each dish. Because *T. denticola* is proteolytic, the bacteria were pretreated with PMSF or TLCK, which are known to inhibit its chymotrypsin-like and trypsin-like protease activities respectively, as described previously (Baehni et al., 1992) prior to adding them to the HGF monolayers. The effect of pretreatment of the OM extract with PMSF was determined by the inositol phosphate assay.

Procedural controls included (1) the omission of LiCl leading to a reduction in the amount of accumulated inositol phosphates due to uninhibited cellular phosphatase activity, and (2) testing the effects of ethanol (the myoinositol solvent) in the IP assay.

Results

Time course for F-actin depolymerization as determined by stress fiber assay and by confocal microscopy.

As determined by the dichotomous stress fiber assay, the normal stress fiber patterns of HGF were progressively altered with time in the presence of *T. denticola* ATCC 35405. By 140 min, 90% of the HGF exhibited an altered stress fiber pattern as compared with 35% for the control bacteria-free HGF (Fig. 6). A significant difference between the percentage of altered stress fibers in the bacteria-challenged and bacteria-free HGF was reached by 60 min [$p < 0.001$ according to the chi-square test for two independent groups with dichotomous data (% of cells showing altered stress fibers in bacteria-challenged vs. bacteria-free HGF) at 60 min]. Under a similar assay condition, *Streptococcus sanguis*, which is not considered as a periodontal pathogen, yet is common in dental plaque, causes neither alterations in stress fiber pattern nor HGF detachment (Baehni et al., 1992).

To analyze changes in F-actin distribution in response to *T. denticola* over time, HGF were stained with rhodamine-phalloidin and optically sectioned (at 1 μm optical steps) by confocal microscopy. A typical

Figure 6. *T. denticola*- induced stress fiber alteration in human gingival fibroblasts. HGF without Td (control) or incubated with Td (Td-treated HGF) at 37 C. At 20 min intervals, cells were fixed and stained with rhodamine phalloidin. Two hundred cells per time point for each treatment were randomly chosen and stress fiber patterns scored dichotomously as either showing normal or altered pattern, according to preset criteria. This figure is representative of one other experiment that gave similar results.

comparison between the actual fluorescence values from one single *T. denticola*-treated HGF (exhibiting an altered stress fiber pattern) and a single bacteria-free HGF (exhibiting a normal stress fiber pattern) at time 60 min is illustrated in Fig. 7. The numbers of optical steps required, as shown in Fig. 7, to section the control HGF and the *T. denticola*-challenged HGF from ventral to dorsal surface were twelve and sixteen steps respectively. However the cell height range for normal fibroblasts is only 3 to 5 μm (Gardner, 1992; McCulloch, 1998). Thus the optical sections reported here do not represent independent additive sections, and the total number of optical sections should not be equated to actual cell height. In my experiment, after the optical sectioning was completed, the sections were then arbitrarily grouped into thirds from the ventral to dorsal surface. The total fluorescence for each HGF cell was also calculated as the sum of fluorescence intensity measurements in all sections for the cell. The mean total rhodamine fluorescence per cell ($n=30$) was significantly less for *T. denticola*-challenged HGF than for bacteria-free HGF by 60 min and at all subsequent time points (at 60 min: mean $\pm$ standard error of the mean were $2.15 \pm 0.24 \times 10^6$ for Td-challenged; $3.32 \pm 0.34 \times 10^6$ for control, $p < 0.01$ according to the independent, two-sample Student's *t* test) (Fig. 8a, 8b). In terms of the partition of the total fluorescence into thirds, the ventral third of the cell showed the greatest proportion of fluorescence intensity. For Td-challenged HGF, at time 0 min, 71.6%, 22.7%, and 5.7% of the mean total

Figure 7. *T. dentico/a*-induced changes in F-actin distribution profiles in HGF after 60 min. The graphs are the actual values from one single experimental and one single control HGF as typical for the data set.

Figure 8a. Reduction and distribution changes in total fluorescence in HGF induced by *T. denticola* ATCC 35405.

Figure 8b. Maintenance of total fluorescence in control HGF over time.

fluorescence were attributable to the ventral, the middle and the dorsal one-third respectively. By 140 min, the total fluorescence decreased by 52% ($p < 0.001$ according to the independent, two-sample Student's *t* test comparing the mean total fluorescence values from Td-challenged HGF at time 0 and 140 min) (Fig. 8a). The greatest proportional change came at the expense of F-actin in the ventral one-third, where the fluorescence intensity decreased by 34% from the initial level (Fig. 9a). For the control Td-free HGF over the same time period, there were no significant changes in either the mean total fluorescence intensity per cell or the proportional distribution of fluorescence intensity (Fig. 8b, 9b).

Treponema denticola cells were pretreated with proteinase inhibitors PMSF or TLCK to determine whether cell-associated proteinases could account for stress fiber disruption. The concentrations of PMSF (170 $\mu\text{g/ml}$) and TLCK (150 $\mu\text{g/ml}$) used here have been shown previously (Baehni et al., 1992; Ellen et al., 1994b) to be effective in inhibiting chymotrypsin-like and trypsin-like activities of *T. denticola* respectively. PMSF inhibits both the endogenous HGF plasma membrane fibronectin degradation by *T. denticola* and *T. denticola*-induced detachment of HGF from its substratum (Baehni et al., 1992; Ellen et al., 1994b). However neither inhibitor reduced the percentages of HGF with altered stress fibers when compared with untreated control and sham-treated (no proteinase inhibitor treatment but

Figure 9a. A shift in the percentage distribution of the total fluorescence in *T. denticola* treated HGF over time.

Figure 9b. Maintenance of distribution of the total fluorescence in control HGF over time.

centrifuged, as in proteinase inhibitor treated samples) *T. denticola* cells (Fig. 10).

Changes in the percentage of cells with altered stress fibers, as determined by the dichotomous stress fibers assay, were in good agreement with the fluorescence intensity measured by confocal microscopy. Regression analysis was used to determine the correlation between data sets represented by the mean fluorescence per cell by confocal microscopy and the mean percentage of cells with altered stress fibers at 6 time points. The correlation coefficient for the two outcomes (the mean fluorescence per cell versus the percentage cells with altered stress fiber) was - 0.89 ($p < 0.01$).

Inositol phosphates in HGF challenged with the type strain of *T. denticola*

Procedural controls.

Procedural controls using *T. denticola* ATCC 35405 were used to verify methods and to partially characterize the responsiveness of the IP pathway. Lithium is an inhibitor of the intracellular enzyme inositol monophosphatase, which plays a major role in the recycling of inositol phosphates (Berridge and Irvine, 1989; Manji et al., 1995). Treatment of cells with LiCl at 10mM concentration allows an accumulation of inositol monophosphates in response to agonist stimulation of [³H]-myoinositol-

Figure 10. Lack of effect of proteinase inhibitors on the disruption of stress fibers induced by *T. denticola*. CIM, HGF challenged with bacteria-free carbon dioxide independent medium; Td, HGF challenged with *T. denticola* ATCC 35405; PMSF, TLCK, HGF challenged with either PMSF- or TLCK-pretreated *T. denticola*; Td-Sham, HGF challenged with *T. denticola* cells incubated in proteinase inhibitor-free buffer and centrifuged identically to samples containing proteinase inhibitors. 54

labeled cells (Martin, 1986). Deletion of LiCl reduced the amount of radiolabeled IPs recovered for both the 1% FBS background control and the 15% FBS positive control. HGF incubated with either 1% or 15% FBS in the presence of 10.0 mM LiCl led to an increase in radiolabeled IPs of 125% and 240% respectively over 60 min. In contrast, parallel samples for which LiCl was deleted yielded negligible radiolabeled IPs at both the beginning of the assay (10% and 14% of the 1% FBS + LiCl and 15% FBS + LiCl controls, respectively) and at 60 min (6% and 7% of the controls). These results verified that incubation of HGF with LiCl indeed allowed the accumulation of radiolabeled inositol monophosphates. This is consistent with the known mechanism of action of Li⁺ on monophosphatase (Manji et al., 1995; Martin, 1986; Berridge and Irvine, 1989). An additional procedural control was to test the effect of ethanol, which was used as a solvent for the myoinositol, as a potential agonist. Ethanol at its assay concentration led to an insignificant change in accumulated IPs over 60 min.

The effects of *T. denticola* ATCC 35405 (type strain) and other strains and related species on IPs recovered in the extracts of HGF was measured over 60 min relative to the effect of background control (1% FBS) and a strongly positive control (15% FBS). The value of IP recovered from HGF at time 0 min varied from experiment to experiment due to the variations in the amount of [³H]-myoinositol actually incorporated. During experiments,

any manipulation of LiCl-treated HGF, even just by washing them, normally causes increases in accumulation of IPs. Therefore, IPs from HGF incubated with the 1% FBS background control increased with time. Following Ruchskowki et al. (1992), we used the 1% FBS background control as the standard comparison. Considering data from 11 separate experiments, the percentage increase in radiolabeled IP for the 1% FBS background control was $212.3 \pm 14.4\%$ (mean $\pm$ S.E.; range 107.8 - 287.6; $p < 0.01$ according to the independent, two-sample Student's t test comparing the mean cpm values for 1% FBS background control at 0 and 60 min). Challenge by *T. denticola* ATCC 35405 resulted in a diminished IP accumulation as evidenced by the consistently lower percentage increase in the concentration of radiolabeled IPs in *T. denticola*-treated HGF in the presence of 1% FBS (mean $\pm$ S.E., $143.1 \pm 13.9\%$; range, 64.7 - 210.4; $p > 0.05$ comparing samples at 0 and 60 min). The increase for 15% FBS was usually several-fold greater on each assay occasion (The percent increase may vary with different batches of FBS from different company suppliers, data not shown.). The % increase for the 15% FBS positive control was $416.3 \pm 49.1\%$ (mean $\pm$ S.E.; range 245.5 - 837.9; $p < 0.001$ comparing samples at times 0 and 60 min). At 60 min, the mean IP concentration for the 15% and 1% FBS controls were significantly different from one another ($p < 0.01$). Over the same time period, the IP concentration in the *T. denticola*-treated HGF was significantly lower ($p < 0.05$) than that for both

1% and 15% FBS controls.

FBS contains a complex mixture of potential agonists (eg. PDGF, EGF) in undefined concentrations that can act cooperatively in stimulating the IP pathway. In order to reduce the complexity in FBS, we tested the effect of *T. denticola* ATCC 35405 on the response of [³H]-myoinositol labeled HGF to ATP over a time period of one hour in two separate experiments. At 60 min, ATP stimulated a 420% increase in radiolabeled IPs. When *T. denticola* and ATP were added simultaneously, the increase in IPs was slightly less, 336%. Preincubation of HGF with *T. denticola* for 30 min prior to addition of ATP (total 90 min experiment) led to a 60 min increase in IPs of 260% versus 380% for ATP alone (average of quadruplicates), an approximately 30% reduction in IP response.

Inositol phosphates in HGF challenged with additional treponemes

After 60 min, the total recovered radiolabeled IPs had increased approximately 7-fold in the 15% FBS-treated HGF positive controls in these experiments (Table 1). In contrast, the percent increase in recovered [³H]-labeled IPs over the same 60 min incubation period was lower for all samples containing *T. denticola* cells (various strains in the presence of 1%

Table 1. Effect of various strains of *T. denticola* and other species of oral treponemes on inositol phosphates in HGF.

Species, strain	<u>Relative cpm [³H]-Inositol Phosphates^a</u>		<u>% Change</u>
	0 min	60 min	0-60 min
1% FBS (bacteria-free control)	100	290	+ 190 %
+ <i>T. denticola</i>			
ATCC 35405	140	200	+ 43 %
ATCC 35404	115	182	+ 58 %
ATCC 33520	85	108	+ 27 %
e'	121	196	+ 62 %
e	108	272	+ 152 %
+ <i>T. socranskii</i>			
ATCC 35536	126	223	+ 77 %
+ <i>T. vincentii</i>			
ATCC 35580	130	174	+ 34 %
15%FBS (bacteria-free control)	128	901	+ 690 %

a. Values for cpm in [³H]-inositol phosphates relative to 1% FBS + LiCl control at beginning of experiment, time 0 min (100%); average of duplicates. For example, the mean value of the 1.0% FBS control at 0 min, 589 cpm, rose to 1707 cpm at 60 min. The 15% FBS control value at 0 min, 753 cpm, rose much more, to 5307 cpm at 60 min. For HGF treated with *T. denticola* 35405, the value at 0 min, 826 cpm, rose less to 1178 cpm at 60 min.

FBS) than that found for the bacteria-free 1% FBS background control. Similar results were found for *T. socranskii* ATCC 35536 and *T. vincentii* ATCC 35580 (Table 1). Thus *T. denticola*, *T. socranskii*, and *T. vincentii* were not only unable to stimulate the IP pathway, they may actually interfere in step(s) in the pathway's response to stimulation from manipulation in a background level of 1% FBS.

Inositol phosphates in HGF challenged with the OM extract

The outer membrane (OM) extracts were prepared from *T. denticola* ATCC 35405 and used instead of the whole bacteria in the HGF challenge assay. Similar to the bacteria, the OM extract yielded a concentration-dependent reduction in labeled IPs relative to the 1% FBS background control (Table 2). Only at a 1/16 dilution was the low level increase in radiolabeled IPs for the OM extract plus 1% FBS comparable to that for the 1% FBS alone. Whole cells of *T. denticola* ATCC 35405 and the OM extract were pretreated with PMSF or TLCK prior to the assay to determine their effects on diminished IP generation. Neither proteinase inhibitor had an effect on the reduction in the yield of radiolabeled IPs when HGF were incubated with 1% FBS and whole cells of *T. denticola* ATCC 35405 (Table 2). Likewise, there was little difference in the relative concentrations of radiolabeled IPs between HGF exposed to 1% FBS and PMSF-pretreated or

Table 2. Inositol phosphate response to *T. denticola* ATCC 35405 and the outer membrane extract.

Assay sample	Relative cpm in [³ H]-Inositol Phosphates ^a		% Change
	0 min	60 min	0-60 min
1% FBS (bacteria-free control)	100	254	+ 154 %
+ whole 35405	94	169	+ 79 %
+ whole 35405/PMSF ^b	120	170	+ 42 %
+ whole 35405/TLCK ^b	100	177	+ 77 %
15% FBS (bacteria-free control)	123	459	+ 273 %
1% FBS (OM-free control)	100	161	+ 61 %
+1/4 OM ^c	117	119	+ 2 %
+1/4 OM/PMSF _b	81	92	+ 11 %
+1/8 OM	96	128	+ 33 %
+1/8 OM/PMSF	75	94	+ 25 %
+1/16 OM	113	209	+ 85 %
+1/16 OM/PMSF	94	207	+ 120 %
15% FBS (OM-free control)	100	307	+ 207 %

a. Values for cpm in [³H]-inositol phosphates relative to 1% FBS + LiCl background control at beginning of experiment, time 0 min (100%); average of duplicates.

b. *T. denticola* cells or outer membrane extract were pretreated with either 170 µg/ml PMSF to inhibit chymotrypsin-like or 150 µg/ml TLCK to inhibit trypsin-like proteinase activity.

c. 1/4 dilution of *T. denticola* ATCC 35405 outer membrane extract.

untreated OM extract (Table 2).

Discussion

Upon exposure to *Treponema denticola*, cultured human gingival fibroblasts undergo profound actin rearrangement and perturbation of actin-modulating signaling pathways prior to and concomitant with the more delayed effects of cell rounding and detachment from the substratum. By establishing a time course for stress fiber disruption, F-actin depolymerization and the recovery of IP pathway activation products, this investigation provides the opportunity to relate temporally the fluctuations in selected, relevant second messengers to actin rearrangement.

Confocal microscopy was used to quantify fluorescence intensity in optical sections of individual HGF in which the F-actin, but not G-actin, was labeled with the fluorescent compound rhodamine phalloidin. In this study, the greatest percentage of fluorescence in normal HGF was detected in the ventral third of the cell (Fig. 9a). This is consistent with other published observations (Small, 1988) showing the ventral location of stress fibers in well spread out fibroblasts. In treponeme-challenged HGF, a significant decrease in whole cell F-actin and a temporally related proportional shift in F-actin from the ventral third to the middle was detected (Fig. 8a, 9a). This reduction in F-actin integrity correlated with a high percentage of HGF with perturbed stress fibers at the end of the assay period, as observed in

dichotomous stress fiber assay (Fig. 6). The finding of reduced whole cell fluorescence reported here may, at first, seem to differ from our previously published observation of an initial increase in total fluorescence (Baehni et al., 1992). The inconsistency may be explained by differences in the methods used. In this study, optical sections of individual single cells were examined to provide more detailed information. This is in contrast to the study of Baehni et al. (1992), where a group of cells in a fixed area of a monolayer was studied using microscopic spectrofluorimetry. With spectrofluorimetry, substantial diminution of fluorescence intensity in the cell periphery may be masked by the more intense emissions of bright fluorescence that condense around the nuclei of cells (Yang et al., 1998).

In this study, *T. denticola*-challenged HGF produced a diminished IP yield when compared with the background level of 1% FBS-stimulated HGF. This reduction coincided temporally with the detection of significant differences in total F-actin and stress fiber integrity between control and *T. denticola*-treated fibroblasts. Both actin and IP recovery were affected significantly within 60 min of bacterial challenge. Thus, some of the inhibitory effects of *T. denticola* on the IP response could precede the more downstream rearrangement of cytoskeletal proteins leading ultimately to HGF detachment from the substratum (Baehni et al., 1992). Since the IP and calcium signaling pathways are interrelated, one would expect that

disturbance in the IP pathway could cause subsequent changes in intracellular Ca^{2+} concentrations. This is what was reported by Ko et al. (1998) on the disturbance by *T. denticola* outer membrane (OM) of HGF internal calcium release, ligand-gated and calcium release-activated calcium channels, and related mechanosensitive cation fluxes. They demonstrated in resting HGF loaded with Fura-2 that OM treatment dramatically increased the spontaneous intracellular calcium oscillations for 20-30 min followed by a complete blockade of calcium oscillations. Also, OM extract partially inhibited ATP-induced and thapsigargin-induced calcium release from intracellular stores. In addition, using HGF loaded with collagen-coated magnetic beads to stimulate integrin-dependent calcium release, magnetically generated tensile force induced a greater than three-fold increase of intracellular calcium above baseline. Such an increase was greatly reduced, in a time- and dose-dependent fashion, in HGF pretreated with OM extract. Thus, Ko et al. (1998) concluded that *T. denticola* outer membrane can inhibit both release of calcium from internal stores and uptake of calcium through channels in the plasma membrane.

Precisely how metabolically active membrane phospholipids affect the organization of the actin cytoskeleton remains unknown. The majority of published studies emphasize the assembly aspect of F-actin rather than its disassembly, which could involve different mechanisms (for review, see

Kandzari et al., 1996). Hence, only features that shed light on the contribution of components of the inositol phospholipid pathway on F-actin disassembly in a fibroblast system will be highlighted here.

As an essential participant in the IP pathway, PIP_2 interacts with numerous cytoskeletal and actin-binding proteins (such as profilin, gelsolin, α -actinin, vinculin), suggesting that the spatially segregated synthesis and metabolism of PIP_2 can be linked to the focal assembly or disassembly of actin filaments (Fukami et al., 1994; Janmey, 1994). Evidence for a role for tyrosine phosphorylation of $\text{PLC}\gamma_1$ in the growth-factor-induced motile response is provided by experiments on the PDGF and EGF receptors, where mutations of the autophosphorylated residue necessary for $\text{PLC}\gamma_1$ binding leads to an abnormal cytoskeletal response (Fantl et al., 1993). The hydrolytic activity of PLC is also affected by the presence of the actin regulatory protein profilin (Goldschmidt-Clermont et al., 1990), which binds to actin monomers and lowers the concentration of free subunits. Isolated profilin also binds PIP_2 (Lassing and Lindberg, 1985, 1988), which inhibits the monomeric actin-binding activity of profilin (Katakami et al., 1992). Profilin has been associated with microfilaments in the tips of newly formed stress fibers of fibroblasts (Buss et al., 1992). Profilin binds to the substrate PIP_2 and inhibits its hydrolysis by unphosphorylated $\text{PLC}\gamma$. Tyrosine-phosphorylated $\text{PLC}\gamma$ with an altered affinity may compete better with

profilin for PIP₂ binding than the unphosphorylated enzyme (Goldschmidt-Clermont et al., 1991; Wahl et al., 1992). At the same time, as the main step in the IP pathway, tyrosine-phosphorylated PLC γ catalyzes the metabolism of PIP₂, thus reducing the availability of PIP₂ to profilin. This suggests that through the hydrolytic activity of PLC γ , tyrosine-kinase-associated receptors can modulate the interaction between profilin and actin by causing a shift in the steady state, which may delay polymerization and induce depolymerization of actin filaments (Stossel, 1989; Goldschmidt-Clermont and Janmey, 1991).

Subsequent to PIP₂ hydrolysis, there is a rise in cytosolic calcium, which can activate gelsolin. Gelsolin is a monomeric actin-binding protein with actin filament severing activity (McLaughlin et al., 1993). This severing activity is activated by micromolar Ca²⁺ or decreased pH (Lamb et al., 1993), and it enables gelsolin to bind to the side of a long actin filament and cut it into two pieces (Allen and Janmey, 1994). Gelsolin has also been implicated in a complex containing both actin and PLC γ (Banno et al., 1992). Gelsolin has been found to bind to PIP₂ (Janmey and Stossel, 1989). PIP₂-binding to gelsolin is incompatible with actin-binding (Janmey et al., 1987).

The study of the protein Rho, a Ras-related monomeric low molecular

weight GTPase, has provided another link in the mechanism by which tyrosine-kinase-associated receptors control the actin cytoskeleton. Rho proteins cycle between two distinct conformational states---active when GTP is bound and inactive when GDP is bound. GTPase activating proteins (GAPs) increase the rate of hydrolysis of bound GTP thereby inactivating Rho. GAPs can bind to phosphotyrosines on activated tyrosine-kinase-associated receptors. Conversely, guanine nucleotide releasing proteins (GNRPs) stimulate the loss of GDP and the subsequent uptake of GTP from the cytosol and, hence, activate Rho (Alberts et al., 1994). Activated Rho rapidly stimulates stress fiber and focal adhesion formation when microinjected into serum-starved Swiss 3T3 fibroblasts (Ridley and Hall, 1992). Readdition of serum produced a similar response (Ridley and Hall, 1992). This activity was initiated by lysophosphatidic acid, which was isolated from serum-containing media.

Rho is essential for the coordinated assembly of focal adhesions and stress fibers induced by growth factors. The exact mechanism by which Rho acts has not yet been determined. Activated Rho stimulates the contractility of smooth muscle, and it may similarly increase the contractility of fibroblast stress fibers and thereby enhance focal adhesion formation (Chrzanowska-Wodnicka and Burridge, 1992). Perhaps Rho induces the formation of actin stress fibers by activating a

phosphatidylinositol 4-kinase and thereby raising the PIP_2 concentration at the site of focal adhesion, where integrins bind to their extracellular ligand (Chong et al., 1994).

An insight into F-actin disassembly can also be gleaned by studying focal contacts. Focal contacts are highly dynamic discrete regions of the ventral plasma membrane where cells adhere tightly to the substratum. Their structure has been recently reviewed by Jockusch et al. (1995). Stress fibers and their modulating elements, which are anchored at or just next to the cytoplasmic face of the plasma membrane at the sites where the cell interacts directly with the extracellular matrix, are the focus of interest here. Focal adhesion assembly requires a number of cellular reactions to take place with tightly controlled equilibrium of their components. Shifting one component, for example, by microinjection of specific antibodies against vinculin (Westmeyer et al., 1990) or talin (Nuckolls et al., 1992) into tissue culture cells may upset such balance, resulting in the disintegration of the entire structure.

The steps leading to focal adhesion formation have not been entirely worked out. Integrin binding to extracellular ligands may induce changes in the conformation of the cytoplasmic domains or in the relationship of the α and β subunits such that the affinity for cytoskeletal proteins (e.g. talin and

α -actinin) increases. Several lines of evidence indicate that other signals involving kinases and possibly low molecular weight G proteins are also required. In fibroblasts, activation of protein kinase C is required for focal adhesion formation (Woods and Couchman, 1992). In addition, one isoform of PKC has been identified in fibroblast focal adhesions (Jaken et al., 1989). As yet, the targets for phosphorylation by PKC in assembling focal adhesions have not been identified.

With the above interactions in mind, one might suggest several avenues of intervention in the IP pathway by which treponemes may account for depolymerization of the F-actin network and disruption of stress fibers listed below:

(1) Growth factors, such as EGF, work by binding to tyrosine-kinase associated receptors, which then dimerize and are activated by autophosphorylation. The activated receptors in turn activate PLC γ to trigger the IP pathway cascade to induce actin polymerization. Treponemes may degrade agonists or interfere with, as yet unidentified, receptors and prevent their activation, leading to a limitation on PLC activation. The idea of host membrane disturbance was entertained because *T. denticola* ATCC 35405, ATCC 35404, and ATCC 33520 produce a phospholipase C (Siboo et al., 1989). Such bacterial enzymes may degrade host membrane

phospholipids. However, my findings in the present study that IPs were diminished, not increased, suggest that exogenous phospholipase C was not the cause of disturbance under conditions of treponemal challenge in intact HGF.

Secondly, *T. denticola* possesses a number of tissue-destructive enzymes (see Review of the Literature section). For example, *T. denticola* possesses chymotrypsin-like activity (Uitto et al., 1988a), which has been shown to degrade endogenous fibronectin on the surface of HGF (Ellen et al., 1994b). It is possible that these enzymes acting in a similar fashion to in vitro trypsinization of tissue culture cells off substratum. These enzymes might degrade receptors, integrins, the extracellular matrix, and thereby disrupt physiologic signaling pathways. Rees et al. (1977, 1980) had described the loss of stress fibers following trypsin treatment of BHK cells and rat dermal fibroblasts. Indeed Fig. 7 might suggest just such a process at work. However upon a closer examination, the involvement of proteolytic enzymes in causing the observed stress fibers and IP pathway disruption was ruled out based on the relative location of attached treponemes to the fibroblasts, the use of protease inhibitors, and temporal correlations of different disruption events: (a) By scanning microscopy, spirochetes were detected in contact with the dorsal HGF surface. However they were never found on the ventral surface of fibroblasts, near focal contacts, between the

substratum and cell (Baehni et al., 1992). (b) PMSF blocks *T. denticola* chymotrypsin-like activity as well as the downstream effect of HGF detachment from the substratum (Baehni et al., 1992, Ellen et al., 1994b). In this study, PMSF and TLCK neither inhibited stress fiber disruption (Fig. 10) nor reversed the diminished IP pathway effects caused by the whole bacteria (Table 2). Similar lack of reversal by PMSF on OM extract-induced diminished IP response was also observed (Table 2). The OM extract exhibited chymotrypsin-like but not trypsin-like activities. In addition, PMSF did not reverse the inhibition of stretch-induced calcium responses mechanosensitive calcium flux by *T. denticola* (Ko et al., 1998). (c) As reported by Ko et al. (1998) at the single cell level, the OM extract induced various intracellular calcium disturbance, which occurred within a time frame of seconds. The stress fiber and IP disruption also occurred before 60 min while fibroblast detachment occurred hours later (Baehni et al., 1992). Therefore, taken together, it is unlikely that the diminished IP response in this study was due to chymotrypsin mediated proteolysis of serum-containing agonists or receptors.

(2) Treponemes may block the production of PIP_2 from PI and PIP, or they may stimulate the hydrolysis of protein-bound PIP_2 via a different biochemical pathway other than through an interaction with tyrosine-phosphorylated PLC γ . With less available PIP_2 , more profilin is available to

shift the G- and F- actin equilibrium in favour of disassembly. The existence of an alternative catabolic pathway for PIP₂ via PIP₂ phosphatase was highlighted recently by Sakisaka et al. (1997). From bovine brain, Sakisaka et al. purified a 150-kDa protein, which has amino acid sequence homologous to that of rat synaptojanin (Sakisaka et al., 1997), an inositol 5-phosphatase (McPherson et al., 1996). This 150-kDa protein can hydrolyze PIP₂ bound to actin-regulatory proteins, which include profilin, cofilin, and α -actinin (Sakisaka et al., 1997). The overexpression of p150 results in the loss of actin stress fibers surrounding the central nucleus area (Sakisaka et al., 1997).

The finding of a still robust IP response in ATP-stimulated HGF that had been pretreated with *T. denticola* suggests that *T. denticola* does not totally block crucial steps in the IP pathway. This is consistent with the general principle of redundancy regulating most signaling pathways, in which there are usually various ways to activate a pathway. When the main IP pathway is blocked, triggering of the pathway by other means may still have been possible. A case in point, the coincubation of HGF with *T. denticola* in the presence of 15% FBS, which alone stimulated a vigorous IP response, resulted in vigorous IP responses above background, instead of the *T. denticola*-induced suppression (data not shown). One explanation is that many growth factors, such as PDGF, EGF, and lysophosphatidic acid, which

are present in FBS, are able to stimulate phospholipase D (PLD) (Kiss, 1992; van der Bend et al., 1992). PLD hydrolyzes phosphatidylcholine into phosphatidic acid and choline (Billah et al., 1991). Phosphatidic acid can be converted into DAG by phosphatidic acid hydrolase, and DAG activates PKC. Thus, while *T. denticola* might block one pathway, an alternative agonist could have accounted for a substantial increase in IPs.

Cytoskeletal disruption and a weak IP response to *T. denticola* are apparently opposite activities to those reported for several enteric and periodontal pathogens (see Review of the Literature). Some enteropathogens stimulate F-actin polymerization as a critical step in signaling their own uptake into host cells, and others stimulate the accumulation of F-actin immediately adjacent to the area of intimate adhesion to tyrosine-phosphorylated host cell membrane receptors (Dytoc et al., 1994; Finlay and Cossart, 1997; Finlay et al., 1992; Rosenshine et al., 1996). Increases in both total IPs (Foubister et al., 1994, Ruschkowski et al., 1992) and specifically IP₃ (Dytoc et al., 1994) follow exposure of epithelial cells to enteric pathogens. The method used in this study to quantify IPs in comparison to a 1% FBS background control was the same as that used to demonstrate increased IP fluxes associated with host cell invasion by *Salmonella typhimurium* (Ruschkowski et al., 1992). Therefore, our affirmation that *T. denticola* did not stimulate increases in IP

concentrations in the period leading up to F-actin disruption was not unexpected.

Conclusion

In light of the diminished inositol phosphate response and the accompanying disruption of filamentous actin induced by oral spirochetes on gingival fibroblasts, some detrimental effects caused by these bacteria on the periodontium can be considered. The fibroblast is the predominant cell type in the soft connective tissues of the periodontium and, consequently, plays a central role both in normal homeostasis and in wound repair. The principal functions of fibroblasts are in the synthesis, secretion and modulation of the fibrous and non-fibrous connective tissue proteins and, through these molecules, the provision of the essential physical attributes and biological properties of the connective tissues (for reviews, see McCulloch and Bordin, 1991; Bartold, 1991). The functions of fibroblasts are influenced by various growth factors (Postlethwaite and Kang, 1988), which can stimulate phosphatidylinositol turnover, cytoskeletal rearrangement and the expression of proto-oncogenes. As has been suggested in the present study, the presence of treponemes or their products can render the gingival fibroblasts non-responsive to bioactive regulating factors and prone to cytoskeletal abnormalities which may result in an imbalance in the development, maintenance, pathological alterations, and wound healing in the periodontium.

In this investigation, I addressed neither the underlying mechanisms nor the microenvironment in which these events may take place. Hence the possible relationships between IP concentration, $[Ca^{2+}]_i$ fluctuations, and F-actin location and integrity are limited to interpretation of temporal, not mechanistic, data on a whole cell level. Therefore, future studies should seek to identify individual *T. denticola* components that contribute to host actin perturbation and to determine the specific mechanisms by which they affect actin-regulating intracellular messengers.

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