Regulation of the Phosphorylation of Human Pharyngeal Cell Proteins by Group A Streptococcal Surface Dehydrogenase: Signal Transduction between Streptococci and Pharyngeal Cells

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Summary

Whether cell-to-cell communication results when group A streptococci interact with their target cells is unknown. Here, we report that upon contact with cultured human pharyngeal cells, both whole streptococci and purified streptococcal surface dehydrogenase (SDH) activate pharyngeal cell protein tyrosine kinase as well as protein kinase C, thus regulating the phosphorylation of cellular proteins. SDH, a major surface protein of group A streptococci, has both glyceraldehyde-3-phosphate dehydrogenase and ADP-ribosylating enzyme activities that may relate to early stages of streptococcal infection. Intact streptococci and purified SDH induce a similar protein phosphorylation pattern with the de novo tyrosine phosphorylation of a 17-kD protein found in the membrane/particulate fraction of the pharyngeal cells. However, this phosphorylation required the presence of cytosolic components. NH_2-terminal amino acid sequence analysis identified the 17-kD protein as nuclear core histone H3. Both phosphotyrosine and phosphoserine-specific monoclonal antibodies reacted with the 17-kD protein by Western blot, suggesting that the binding of SDH to these pharyngeal cells elicits a novel signaling pathway that ultimately leads to activation of histone H3–specific kinases. Genistein-inhibitable phosphorylation of histone H3 indicates that tyrosine kinase plays a key role in this event. Treatment of pharyngeal cells with protein kinase inhibitors such as genistein and staurosporine significantly inhibited streptococcal invasion of pharyngeal cells. Therefore, these data indicated that streptococci/SDH-mediated phosphorylation plays a critical role in bacterial entry into the host cell. To identify the membrane receptor that elicits these signaling events, we found that SDH bound specifically to 30- and 32-kD membrane proteins in a direct ligand-binding assay. These findings clearly suggest that SDH plays an important role in cellular communication between streptococci and pharyngeal cells that may be important in host cell gene transcription, and hence in the pathogenesis of streptococcal infection.

Group A streptococci (*Streptococcus pyogenes*) cause a variety of human diseases, the most common of which is acute pharyngitis. Recently, the increasing incidence of rheumatic fever and severe group A streptococcal–related invasive infections, including a toxic shock–like syndrome, has posed special concern worldwide (1, 2). While the cause of this reemergence is unknown, it is apparent that microorganisms have developed novel strategies to interact with host cell receptor molecules and to regulate intracellular signaling pathways of host cells to induce their own adherence, colonization, and internalization (3–7). Bacterial surface proteins, while serving as ligands to interact with host cell receptors, play a crucial role in signaling events (7, 8). Although many proteins have been described on the surface of group A streptococci (9), none have been shown to play a direct role in cellular signaling.

We showed earlier that streptococcal surface dehydrogenase (SDH),[1] a 35.8-kD protein, is structurally and functionally related to glyceraldehyde-3-phospate dehydrogenase (GAPDH) (reference 10), has multiple binding activity to a variety of mammalian proteins (10), and is an ADP-ribosylating enzyme (11). SDH is commonly found on the surface of all serological types of group A streptococci and those of other serological groups, implicating its importance for streptococcal survival (10). Recently, a GAPDH molecule was also reported to be present on the surface of

[1] *Abbreviations used in this paper:* GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 1G2, PSR-45-antiphosphotyrosine monoclonal antibody; M/P, membrane/particulate; PVDF, polyvinyldifluoride; SDH, streptococcal surface dehydrogenase.

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Streptococcus pneumoniae (12). Several recent studies have indicated new roles for GAPDH in fundamental yet unrelated mammalian cell processes such as DNA repair, translational control of gene expression, DNA replication, and endocytosis (13). A report by Doucet and Tuana (14), showing the ability of GAPDH to bind to low molecular weight GTP-binding proteins, suggested that GAPDH may play an important role in membrane communication. While much is already known at the molecular level regarding the perturbation of host cell function by overtly invasive Gram-negative bacteria responsible for enteric or urogenital infections (3–7, 15, 16), similar information is unavailable for the Gram-positive pathogens in general and *S. pyogenes* in particular.

In this study, we examine the effects of the interaction of SDH and intact streptococci on the type and nature of protein phosphorylation of pharyngeal cells. We show that both SDH and streptococci interact specifically with a membrane/particulate (M/P) component of pharyngeal cells, and this, in the presence of cytoplasmic components, determines the protein phosphorylation profile of these cells. We further identify and characterize a 17-kD pharyngeal cell protein that is tyrosine and serine phosphorylated only after cellular interaction with streptococci or purified SDH. In the absence of any published reports on signal transduction events during streptococci–pharyngeal cell interactions, our findings, like those in Gram-negative infections, clearly suggest that signal transduction may play an important role in the pathogenesis of streptococcal disease and, perhaps, other Gram-positive bacterial infections.

Materials and Methods

Materials and Chemicals. M type 6 group A streptococcal strain D471 was from The Rockefeller University collection, and was grown in Todd-Hewitt broth (Difco Laboratories, Inc., Detroit, MI) supplemented with 0.3% yeast extract. SDH was purified from the cell wall extracts of the same strain as previously described (10). [γ-^{32}P]ATP (6,000 Ci/mmol) and [^{125}I]NaI (17 Ci/mg) were obtained from NEN Life Science Product (Boston, MA). Antiphosphotyrosine monoclonal antibody (1G2; 1 mg/ml) was obtained from Boehringer Mannheim (Indianapolis, IN), and antiphosphoserine monoclonal antibody (PSR-45; IgG1) was obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals, unless otherwise mentioned, were obtained from Sigma Chemical Co.

Human Pharyngeal Cells. Detroit 562 (human pharyngeal carcinoma, ATCC CCL-138) and FaDu (Human pharyngeal squamous cell carcinoma, ATCC HTB-43) cell lines were obtained from the American Type Culture Collection (Rockville, MD). They were grown and maintained at 37°C, 5% CO_2/95% air in MEM (GIBCO BRL, Bethesda, MD) supplemented with 1 mM sodium pyruvate and 10% (vol/vol) fetal bovine serum.

Cell Fractionation. Detroit and FaDu pharyngeal cells were grown to confluence, harvested (4×10^7 cells/ml), and lysed in a hypotonic buffer (2 mM imidazole, pH 7.4, containing 5 mM $MgCl_2$, 2 mM PMSF, DNAse [100 μg/ml], and RNAse [50 μg/ml]) on ice for 1 h. Complete cell lysis was achieved by several cycles of freezing and thawing. The resulting lysate was then separated into cytosol and M/P fractions by ultracentrifugation (100,000 *g* for 15 min at 4°C; reference 11). The concentration of both M/P and cytosolic fractions was adjusted to 4 mg/ml and stored at −70°C until further use.

Phosphorylation of Pharyngeal Cell Proteins. Detroit pharyngeal cells (10^6 cells) in a phosphorylation buffer (20 mM imidazole, 20 mM sodium acetate, 2 mM magnesium acetate, 2 mM EGTA, and 2 mM DTT, pH 7.4) were reacted with group A streptococci (4×10^6 CFU), purified SDH (3.5 μg), or purified recombinant M protein (3.5 μg; reference 17) for 10 min at 37°C. At that time, phosphorylation was carried out in the presence of 10 μCi of [γ-P^{32}]ATP at 30°C for another 45 min in a final volume of 250 μl of phosphorylation buffer. A duplicate set of experiments was also carried out in the presence of genistein (100 μM), a tyrosine kinase inhibitor, and/or staurosporine (1 μM), an inhibitor of protein kinase C and other kinases. At the end of the reaction, the samples were quickly centrifuged (12,000 *g* for 1 min at 4°C) and the resulting pellets were suspended in half of the original reaction volume in hypotonic buffer (125 μl) containing 0.5 mM sodium vanadate, snap frozen in dry ice/ethanol to stop the reaction, and then the cells were lysed as described above. The resulting cell lysates were further separated into cytosol and M/P fractions by ultracentrifugation as described above, and were adjusted to a starting reaction volume (250 μl) with SDS-PAGE sample buffer.

A similar separate experiment was also carried out using a cytosol-free M/P fraction in order to understand the role of cytoplasmic contents in protein phosphorylation of the M/P fraction. For this experiment, the M/P fraction (200 μg) as described above was first treated with streptococci/SDH in the presence and absence of genistein and/or staurosporine, and the protein phosphorylation profile was determined as described above. 50 μl of each sample was applied per lane of an SDS-PAGE, and Western blotting on a polyvinyldifluoride (PVDF) membrane was performed as described (10, 11). The PVDF membranes were then stained with Coomassie blue, and the same membranes were subjected to autoradiography to accurately identify, locate, and align the phosphorylated proteins with the Coomassie-stained proteins. Autoradiography of all the gels was carried out using the same exposure time to accurately determine the relative quantitative difference in the protein phosphorylation.

Immunoprecipitation. At the end of phosphorylation experiments, the 17-kD protein was immunoprecipitated from the M/P and cytosolic fractions. Both M/P and cytosolic fractions were suspended in the original reaction volume of an immunoprecipitation buffer (IPB) (250 μl of 10 mM Tris/HCl, 0.25 M NaCl, 1% Triton-X100, 0.5% NP-40, 0.1% SDS, 1 mM EGTA, 1 mM EDTA, and 0.5 mM sodium vanadate), centrifuged (11,000 *g*, 5 min, 4°C), and the soluble supernatants were collected. 10 μg of antiphosphotyrosine antibody 1G2 was added to the soluble fractions and incubated at room temperature for 2 h. 50 μl of 50% preswelled protein A-Sepharose CL-4B suspension in IPB was then added to the resulting immune complex–containing suspension, and was incubated for 1 h at 4°C. Sepharose beads containing the immune complexes were collected by centrifugation, washed several times, suspended in 80 μl of 2× electrophoresis sample buffer containing β-mercaptoethanol, and boiled. Proteins were then resolved on SDS-PAGE, Western-blotted on a PVDF membrane, and stained by Coomassie stain or Ponceau-S stain. The same PVDF membrane was then subjected to autoradiography as previously described (10, 11) to accurately identify the labeled phosphorylated proteins that were immunoprecipitated.

NH_2-terminal Protein Sequencing and Peptide Mapping. NH_2-terminal amino acid sequence of the immunoprecipitated 17-kD pro-

tein was determined as previously described (10, 18). In brief, the immunoprecipitated 17-kD protein was resolved on a PVDF membrane by SDS-PAGE and Western blotting. The protein was visualized by staining with 0.1% Ponceau-S (Sigma Chemical Co.) in 1% acetic acid. The section of the membrane containing the protein band was excised, destained with distilled water, and subjected to automated Edman degradation. All microsequence analyses were performed at the Protein/Biotechnology facility of The Rockefeller University.

SDS-PAGE, Western Blotting, and Immunostaining. Phosphorylated pharyngeal cell proteins were resolved on 11% SDS-PAGE, and electrotransferred onto a PVDF membrane as previously described (10, 11). The PVDF membranes were then subjected to autoradiography. For immunostaining, the blots were blocked in 50 mM Tris/HCl, pH 7.4, buffer containing 1% BSA, 0.5% Tween 20, 1% Triton X-100, 0.5% NP-40, 0.25 M NaCl, and 1 mM sodium vanadate for 3 h at room temperature. The blots were then stained separately with 1G2 and PSR-45–specific mouse monoclonal antibodies for 3 h, followed by anti–mouse IgG conjugated with horseradish peroxidase for 2 h at room temperature. The specific stained protein bands were detected by an enhanced chemiluminescence Western blotting detection system (Amersham Corp., Arlington Heights, IL).

Transmission Electron Microscopy. After treatment of confluent cell monolayers on polyethylene terepthalate transwell membranes (0.4 μm, 0.9 cm^2; Becton Dickinson Labware, Franklin Lakes, NJ) or conventional 24-well tissue culture plates with purified SDH (3.5 μg/10^6 cells) for 1 h at 37°C, the monolayers were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. The cells were then postfixed in 1% osmium tetroxide stained in 0.25% uranyl acetate, and further processed for electronmicroscopy in the central biotechnology facility at The Rockefeller University.

Cell Viability and Cytotoxicity Assay. To determine the effect of SDH and streptococcal treatment on the viability of Detroit cells, Detroit cells (10^6) were initially treated with various concentrations of SDH (0.875–14 μg) and group A streptococci (2×10^6–2×10^7 cfu) in a final volume of 250 μl of Dulbecco PBS (D-PBS) or phosphorylation buffer for 1 h at 37°C under constant slow rotation. The cells were then centrifuged, and the cell pellets obtained were stained with 200 μl of Live/Dead Viability/Cytotoxicity Fluorescence Stain containing 4 μM ethidium homodimer and 2 μM calcein AM (Molecular Probes, Inc., Eugene, OR) as per the manufacturers instructions. The cells were then washed twice in D-PBS, and live (green fluorescing) and dead cells (red fluorescing) were counted in a cell counting chamber by fluorescence microscopy.

Streptococcal Adherence and Invasion Assays. Streptococcal adherence to Detroit and FaDu pharyngeal cells was carried out in 24-well tissue culture plates essentially as previously described (19) with some modifications. In brief, wells with confluent cell growth ($0.8–1.2 \times 10^6$ cells/well) were left untreated (control) or treated with tyrosine protein kinase inhibitor (100 μM genistein) and a broad spectrum ser/thr-kinase inhibitor, staurosporine (1 μM), for 15 min before addition of MEM-prewashed overnight culture of group A streptococci (50 bacteria:1 cell), and were incubated in a humidified CO_2 incubator at 37°C for different time intervals (1–3 h). Nonadherent bacteria were removed by repeated washing, and the Detroit cells with adherent bacteria were lysed in 500 μl of cell lysis solution (MEM containing 0.1% trypsin, 1% Triton X-100, 50 μg/ml DNAse, and 25 μg/ml RNAse). The lysates were then vortexed vigorously, appropriately diluted, and the cell-associated group A streptococci were counted as CFU by the blood agar pour-plate method. The maximum streptococcal adherence to Detroit cells was seen by 3 h of incubation. Incubation of streptococci with FaDu cells for more than 1.5 h resulted in detachment of cells from the culture plates, leaving them unsuitable for the adherence and invasion assays. Hence, the adherence and subsequent invasion assay with Detroit cells were carried out for 3 h, and those with FaDu were carried for 1h 15 min.

For the bacterial invasion assay, the cell-associated bacteria in the 24-well tissue culture plates were treated with gentamicin (100 μg/ml) and penicillin (10 μg/ml) for 90 min at 37°C, washed (3×) with MEM, and the internalized streptococci were released using cell lysis solution. The resulting lysates were then processed for CFU as described above.

Three sets of such experiments each with four to six wells/parameter were carried out. Mean values of CFU were calculated from the average values obtained from each set of experiments for statistical analysis.

Ligand Blot Assay. The crude lysates of Detroit and FaDu pharyngeal cells were centrifuged (1,000 *g* for 10 min at 4°C) to pellet the particulate components such as organelles and nucleosomes. The supernatants thus obtained was then ultracentrifuged (100,000 *g* for 1 h at 4°C) to obtain plasma membrane and cytosolic fractions. The proteins in the plasma membranes of Detroit and FaDu cells were separated on 11% SDS-PAGE gels, Western blotted on a PVDF membrane, blocked (50 mM Tris/HCl, 0.5% Tween 20, 0.5% NP-40, 0.15 M NaCl, 0.5% gelatin, 1% BSA, and 0.04% sodium azide) for 3 h, and probed with [^{125}I]SDH (3.0×10^5 cpm/ml, specific activity: 2×10^6 cpm/μg of SDH) for 4 h at room temperature (10). The probed blots were washed several times with washing buffer (half the strength of blocking buffer containing 0.35 M NaCl), dried, and the binding of the labeled SDH was visualized by autoradiography as previously described (10). The specific binding of SDH to pharyngeal membrane proteins was carried in the presence of excess amounts of unlabeled SDH (based on the quantity of ^{125}I-SDH/ml of reaction volume) on an individual PVDF membrane strip containing equal amounts of pharyngeal membrane proteins. The individual blots were processed as described above, and inhibition of the binding of ^{125}I-labeled SDH to pharyngeal membrane proteins was determined by autoradiography.

Results

Since protein phosphorylation and its activation by external stimuli play important roles in the initiation of signaling events, we determined the ability of group A streptococci and purified SDH to induce host cell protein phosphorylation as a result of their binding to human pharyngeal cells, Detroit 562 and FaDu. All phosphorylation experiments with Detroit 562 were carried out in the presence of genistein, a tyrosine kinase inhibitor, and/or staurosporine, a broad spectrum inhibitor of serine/threonine-specific protein kinases including protein kinase C, to determine the nature of streptococcal/SDH-mediated protein phosphorylation.

Streptococcal/SDH-mediated Phosphorylation of Proteins in the M/P Fraction of Intact Pharyngeal Cells. Because of its surface location, SDH may be predicted to directly contact the pharyngeal cell membrane or other cellular components, depending on the stage of pharyngeal infection by streptococci. Hence, we first compared the effect of whole strep-

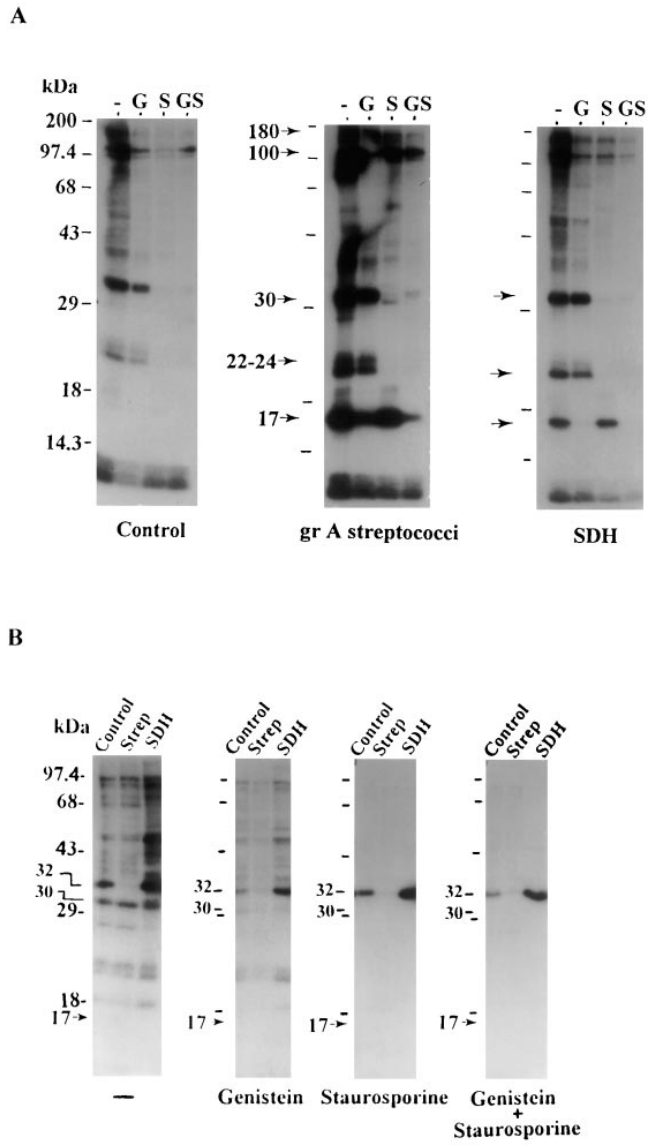


Figure 1. ^{32}P-labeled proteins of the Detroit pharyngeal cell M/P fraction. Autoradiographs showing (*A*) ^{32}P-labeled proteins of the M/P fraction after phosphorylation of intact pharyngeal cells, and (*B*) ^{32}P-labeled proteins after phosphorylation of the isolated M/P fraction, each then treated with group A streptococci (gr A streptococci), or purified SDH. After treatment, phosphorylation was carried out in the presence of [γ-^{32}P]ATP. Genistein (*G*), staurosporine (*S*), or both (*GS*) were included in the reaction mixtures to determine the amino acid site specificity of the phosphorylation modifications. Phosphorylation of pharyngeal cells without any prior treatment served as the control. Controls in which bacteria alone were processed under similar conditions released no phosphorylated proteins in the soluble fraction (not shown). Each lane received ~40 μg of total protein. Figures on the left side are the molecular mass (*kDa*) of standard prestained proteins (GIBCO BRL). Major phosphorylated proteins are indicated by molecular mass (*kDa*).

tococci or purified SDH on the phosphorylation of proteins in intact Detroit cells, in the presence of [γ-^{32}P]ATP. After phosphorylation, the cells were lysed, and the resulting cell extract was then separated into cytosolic and M/P fractions, the latter containing the plasma membrane, organelles, and nucleosomes. In the absence or presence of whole streptococci/SDH, several proteins in M/P fraction were phosphorylated at varying intensities (Fig. 1 *A*), while the incorporation of ^{32}P in cytosolic proteins was found to be very weak (data not shown). In the presence of intact streptococci or SDH, phosphorylation of several proteins in the M/P fraction was enhanced, with greater incorporation of ^{32}P in the presence of streptococci than SDH (Fig. 1 *A*). Interestingly, phosphorylation of a 17-kD protein in this M/P fraction occurred only after its interaction with whole streptococci or SDH. While SDH-mediated phosphorylation of the 17-kD protein was completely inhibited by the tyrosine kinase inhibitor, genistein, and significantly inhibited by the mixture of 100 μM genistein and 1 μM staurosporine, it was partially inhibited in the case of whole streptococci–mediated phosphorylation. Staurosporine alone also reduced the streptococcal/SDH-mediated phosphorylation of the 180-kD, 100-kD, and completely inhibited that of the 30-kD and 22–24-kD pharyngeal cell proteins. These results indicate that pharyngeal cell phosphorylation mediated by whole streptococci is likely induced by SDH via the activation of both protein tyrosine kinase and other kinases, including protein kinase C.

Role of Cytosolic Components in the Phosphorylation of Proteins in the M/P Fraction of Intact Pharyngeal Cells. In the previous experiment, the phosphorylation reaction was performed in intact cells before separation into M/P and cytosol. To determine if cytosolic components play any role in the phosphorylation of the proteins in the M/P fraction, the cytosol-free M/P fraction, obtained by ultracentrifugation of the Detroit cell extract, was used for streptococci/SDH-mediated phosphorylation. Our results show that the M/P fraction–associated 17-kD protein was not phosphorylated in the presence of streptococci or SDH under these conditions (Fig. 1 *B*), indicating that the enzymes present in the cytosolic compartment are responsible for the ultimate phosphorylation of the 17-kD protein.

In addition to the 30-kD protein that was phosphorylated in intact cells (Fig. 1 *A*), a new 32-kD protein was also found to be phosphorylated when the cytosol-free M/P fraction was used in the phosphorylation reaction (Fig. 1 *B, Control*). While the phosphorylation of this 32-kD protein was unaffected in the presence of SDH, it was inhibited when whole streptococci were used (Fig. 1 *B, Strep*). In the presence of genistein and/or staurosporine, the phosphorylation profile of the 32-kD protein was unchanged, while that of the 30-kD protein was inhibited (Fig. 1 *B*). These data suggest that streptococci may have the capacity to induce specific dephosphorylation events that cannot be mediated by SDH alone.

Cell Specificity for Streptococci/SDH-mediated Protein Phosphorylation. To determine the ability of streptococci/SDH to regulate phosphorylation of cellular proteins of other human pharyngeal cell lines, we used another established pharyngeal cell line, FaDu, and compared the protein phosphorylation profile with that of Detroit cells. The results (Fig. 2) show a similar phosphorylation pattern in control untreated FaDu cells. FaDu cells treated with intact

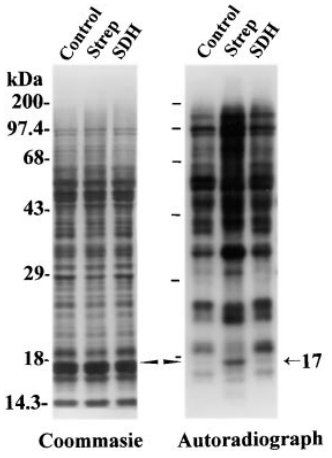


Figure 2. ^{32}P-labeled proteins of the FaDu pharyngeal cell M/P fraction. Autoradiographs showing ^{32}P-labeled proteins of the M/P fraction after phosphorylation of intact pharyngeal cells. FaDu cells were prepared, processed, and treated with group A streptococci and purified SDH as described in Fig. 1.

streptococci show a marked increase in protein phosphorylation in the M/P fraction with a de novo phosphorylation of the 17-kD protein as observed in Detroit cells (Fig. 1). However, unlike the Detroit cells (Fig. 1), the de novo incorporation of labeled ^{32}P in the 17-kD protein of FaDu cells in the presence of purified SDH increased only slightly. These data confirm the role of intact streptococci in regulating phosphorylation of pharyngeal cell proteins, and indicate that the interaction of streptococci with FaDu cells may be similar to that found with Detroit cells. The interaction of purified SDH with FaDu cells leading to relatively less incorporation of labeled ^{32}P in the 17-kD protein suggests that this interaction may be cell-type dependent, and, accordingly, may result in different signaling pathways.

To further determine the cell specificity of SDH-mediated phosphorylation, similar experiments were performed with intact Chang (human conjunctiva), Chinese hamster ovarian, human epithelioid carcinoma, or human hepatocellular epithelial cell lines. SDH did not affect the protein phosphorylation pattern in the M/P fraction of these cells (data not shown) as seen with Detroit and FaDu cells (Figs. 1 and 2), and in no case was a 17-kD protein phosphorylated. As a parallel control, M protein, an α-helical coiled-coil surface protein on group A streptococci (20) was used in the Detroit cell phosphorylation experiments, and was found to have no effect on their protein phosphorylation pattern (not shown).

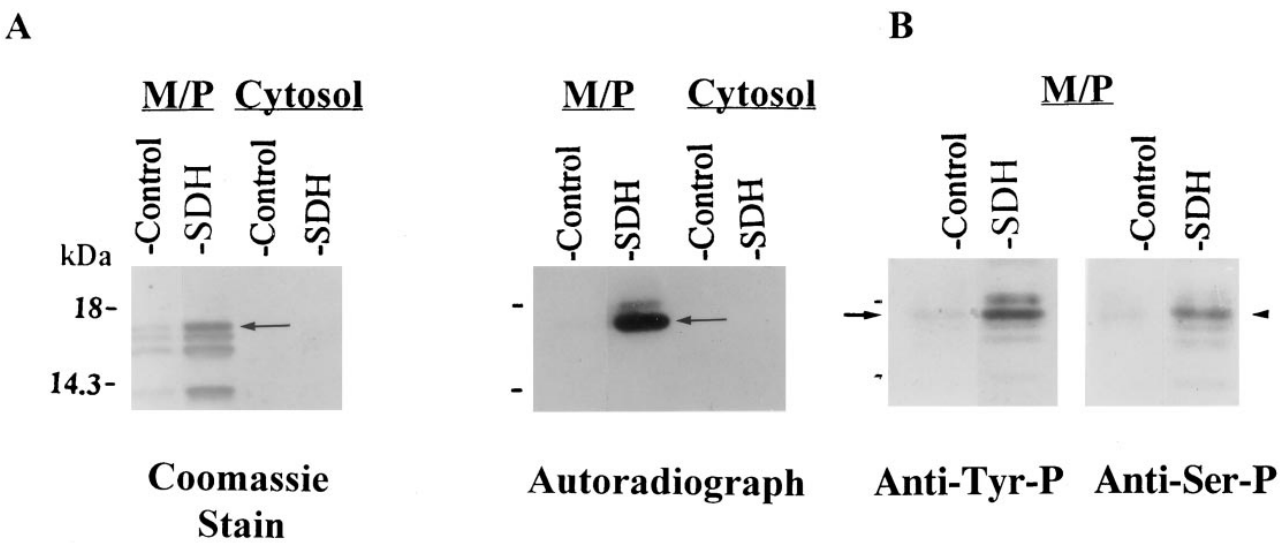
Isolation and Characterization of the 17-kD Phosphorylated Protein. To isolate SDH-mediated tyrosine phosphorylated pharyngeal cell proteins, intact pharyngeal cells were treated with SDH (as in Fig. 1), and [^{32}P]ATP-labeled M/P proteins were immunoprecipitated using antiphosphotyrosine monoclonal antibodies after cell lysis and fractionation. We found that the labeled 17-kD protein was immunoprecipitated along with three other closely migrating nonlabeled proteins (Fig. 3 *A*). To further confirm that the immunoprecipitated 17-kD protein is tyrosine phosphorylated, Western blots containing the labeled 17-kD proteins were reacted with either antiphosphotyrosine or antiphosphoserine antibodies. We found that both monoclonal antibodies bound to the 17-kD protein, with stronger reactivity exhibited by the antiphosphotyrosine antibody (Fig. 3 *A*). These results, while confirming the tyrosine phosphorylation of the 17-kD protein, also revealed that this protein is phosphorylated at serine residues (Fig. 3 *B*).

By superimposing the autoradiograph of a PVDF membrane onto the same membrane in which the labeled 17-kD protein had been stained with Ponceau-S, we precisely identified the protein in the PVDF membrane for excision and NH_2-terminal sequence analysis. NH_2-terminal amino acid sequence revealed a single amino acid at all 15 positions, resulting in the sequence: ^{1}ARTKQTARKSTGGKA^{15} (Fig. 3 *C*). When this sequence was compared to known sequences in the translated GenBank database, 100% identity was found with histone H3 of human, animal, yeast, and plant origins (Fig. 3 *C*). Since histone H3 has previously been implicated in signal transduction events (21–25), our results suggest that it may play an important role in streptococcal/SDH-mediated signaling events in pharyngeal cells. This result also suggests that based on their molecular mass and characteristic migration pattern, the proteins that coprecipitated with the histone H3 protein (Fig. 3 *A*) are likely histone H2A, H2B, and H4 (23, 26).

Ultrastructural Changes in and Cell Viability of SDH-treated Pharyngeal Cells. Protein phosphorylation in general has a significant impact on cell shape, size, and motility (27), and phosphorylation of histone proteins in particular has been shown to result in condensation of chromatin structure (21–23, 28–30). Therefore, we investigated whether we could observe these ultrastructural changes in SDH-treated Detroit cells. Results obtained by transmission electron microscopy of control untreated Detroit cells grown on the surface of Transwell membranes revealed a highly compact monolayer of cells with distinct tight junctions at their basolateral sides towards the apical region. In contrast, using the same parameters as the phosphorylation experiments, Detroit cells treated with SDH showed a clear disruption in this arrangement (Fig. 4 *A*) with a change in shape and size and a significant change in the nucleus-to-cytoplasmic size ratio. Furthermore, chromatin was found to be distinctly condensed within the nucleus when compared with control untreated cells (Fig. 4 *B*). Similar findings were also obtained with nonpolarized Detroit cells grown on polystyrene plates (Fig. 4 *C*). These findings further support the role of SDH-mediated induction of protein phosphorylation in cell morphology, and in particular that of histone H3 in nuclear condensation.

Since nuclear condensation may influence cell viability, we used a highly specific and sensitive cell viability/cytotoxicity assay to determine whether the observed SDH/streptococcal-mediated changes in Detroit cells are cytotoxic. SDH- and streptococcal-treated Detroit cells were not found to be significantly affected (2–5% red fluorescing dead cells) in their staining pattern when compared with control untreated cells, indicating that SDH is not overtly cytotoxic to pharyngeal cells.

Streptococcal Adherence and Invasion. To investigate whether the induction of tyrosine and serine/threonine phosphorylation of pharyngeal cellular proteins can influence the abil-



C.

^{1}A R T K Q T A R K S T G G K A^{15} 17 kDa Pharyngeal Protein

^{1}M A R T K Q T A R K S T G G K A^{16} Human Histone-H3

^{1}M A R T K Q T A R K S T G G K A^{16} Plant, Sea Urchin, Yeast, Chicken, Mouse, Drosophila

Figure 3. Identification of the 17-kD phosphorylated protein. (*A*) Coomassie stain and autoradiograph of a PVDF membrane showing the immunoprecipitated phosphorylated 17-kD protein with antiphosphotyrosine monoclonal antibodies. Detroit cells were treated with SDH, and the phosphorylation reaction was carried out as described in Fig. 1. Pharyngeal cell M/P fraction and cytosolic proteins were then immunoprecipitated with antiphosphotyrosine monoclonal antibodies. Proteins in the precipitate were resolved by 11% SDS-PAGE and electrotransferred to a PVDF membrane. The *arrows* indicate the phosphorylated 17-kD membrane protein closely migrating with three other coprecipitated nonphosphorylated membrane proteins. (*B*) Direct immunostaining of the immunoprecipitated 17-kD protein complex on Western blot. The blots were stained with with antiphosphotyrosine and antiphosphoserine mouse monoclonal antibodies followed by HRP-labeled antimouse IgG conjugate and detected with ECL Western blotting detection reagents. The phosphorylated 17-kD protein as detected by this method is marked (*arrow*). (*C*) NH_2-terminal amino acid sequence of the immunoprecipitated 17-kD phosphorylated protein and its comparison with the NH_2-terminal sequence of human histone H3 and other representative eukaryotic histone H3 proteins.

ity of group A streptococci to adhere and subsequently invade the host cell, these processes were studied in the presence and absence of protein kinase inhibitors (genistein or staurosporine) using both Detroit and FaDu cells. As shown in Fig. 5, streptococci adhered efficiently to Detroit pharyngeal cells ($4.9 \pm 0.5 \times 10^6$ CFU/ well), and this adherence was not significantly affected by treatment with the protein tyrosine kinase inhibitor, genistein, or a broad spectrum protein kinase inhibitor, staurosporine. However, in the presence of these inhibitors streptococcal invasion was significantly inhibited (Fig 5). Similar results were obtained when the adherence and invasion assays were performed using FaDu pharyngeal cells. The lower level of streptococcal adherence to and invasion of FaDu cells reflects the shorter incubation time used for the FaDu-based adherence assay as compared to Detroit cells (1.25 versus 3 h). Longer incubation of streptococci with FaDu cells resulted in detachment of the cells from the tissue culture plates. Both genistein and staurosporine inhibited streptococcal invasion of Detroit as well as FaDu pharyngeal cells by as much as 20-fold. These results indicate that SDH/streptococci-mediated induction of protein tyrosine kinase and protein kinase C are important for streptococcal invasion.

Identification of an SDH-specific Receptor on Pharyngeal Cells. The above findings clearly indicated that the interaction of SDH/streptococci with intact pharyngeal cell surfaces results in coordinated phosphorylation of both cytosolic and membrane proteins, which in turn leads to phosphorylation of histone H3 located in the cell nucleus. Therefore, we attempted to identify the SDH-specific receptor on the sur-

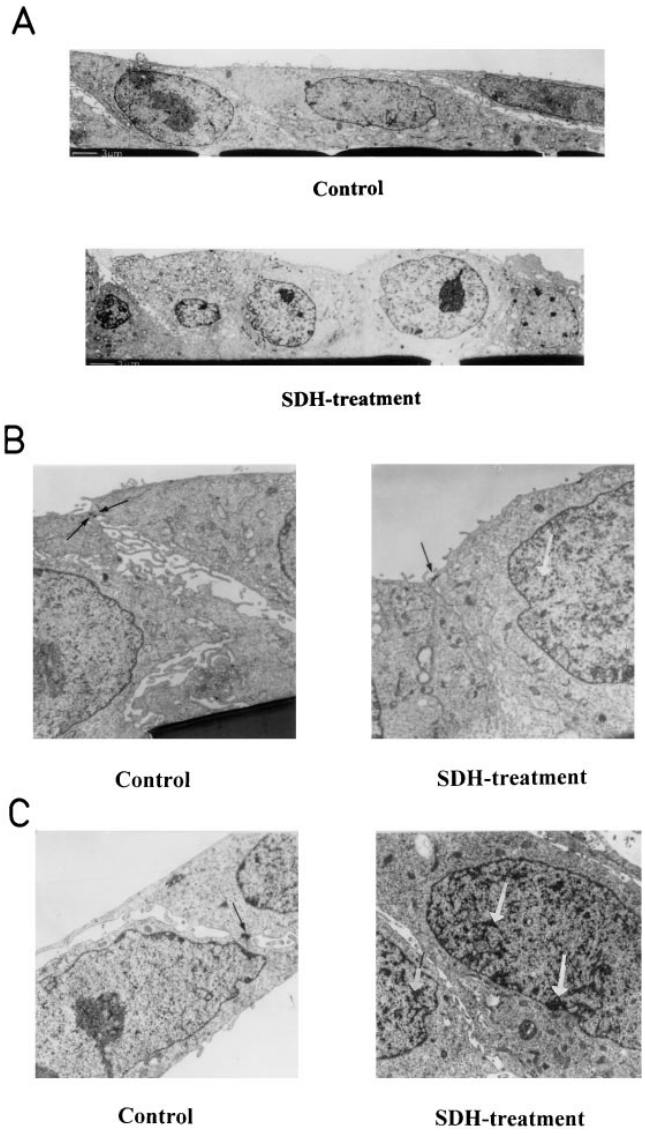


Figure 4. Transmission electron microscopy (TEM) of SDH-treated and -untreated (*Control*) pharyngeal cells grown on Transwell membranes. (*A*) TEM viewed at low magnification (3,250×). (*B*) TEM viewed at higher magnification (6,600×) emphasizing both the nuclear morphology and apical tight junction (*arrow*) of control and SDH-treated pharyngeal cells. The SDH-treated panel shows the nucleus with marked chromatin condensation (*white arrows*). (*C*) TEM of nonpolarized Detroit cells grown on polystyrene plates treated similarly as in *A*. Original magnification, 6,600×. Nonapical tight junction (*arrow*) and chromatin condensation in the nucleus of SDH-treated cells (*white arrows*) are indicated.

face of the pharyngeal cells. To accomplish this goal, plasma membranes were fractionated from Detroit and FaDu cells, and the proteins contained therein were probed with [^{125}I]SDH by a direct ligand binding assay on Western blots. The results revealed that the [^{125}I]SDH bound to Detroit pharyngeal cell membrane proteins at 30 and 32 kD (Fig. 6 *A*). Similarly, only a 32-kD protein of FaDu pharyngeal cell membranes bound to [^{125}I]SDH (Fig. 6 *A*). Binding of ^{125}I-labeled SDH to the 30/32-kD Detroit pharyngeal cell membrane proteins was significantly inhibited by a 40–80 molar excess of unlabeled SDH, suggesting that these proteins may serve as receptor(s) for the SDH molecule (Fig. 6 *B*).

Discussion

The ability of several pathogenic microorganisms to interact with their specific host cells, resulting in a cascade of intracellular signal transduction events, has in recent years generated a great deal of interest. Studies have provided important clues towards understanding the cell biology and biochemical basis of invasion of nonphagocytic cells mainly by enteropathogenic Gram-negative bacteria (3–7, 15, 16). Similar information for Gram-positive bacteria is largely lacking, although pathogenic Gram-positive bacteria, more specifically group A streptococci, are known to cause a variety of serious diseases and show a remarkable tissue tropism for the upper respiratory tract and skin. In this study, we used both the Detroit 562 and FaDu human pharyngeal cell lines to explore those aspects of pharyngeal cell signaling events that are induced by group A streptococci. We report here that this organism and its major surface protein SDH are in fact capable of promoting signal transduction in pharyngeal cells.

Since phosphorylation is the key biochemical event regulating signal transduction in eukaryotic cells, we first investigated the effect of streptococcal interaction on the phosphorylation of pharyngeal cell proteins, and compared this effect with that obtained using purified SDH. We found that whole streptococci and purified SDH induced phosphorylation of several pharyngeal cell proteins. Using intact pharyngeal cells and prefractionated M/P fractions, we were able to show that streptococcal/SDH-mediated phosphorylation of these proteins required cooperation between membrane, cytosolic, and nuclear components. Thus, de novo tyrosine phosphorylation of the nuclear-associated 17-kD protein occurred only after interaction of streptococci or SDH with intact pharyngeal cells (Figs. 1 *A* and 2) or whole cell lysates. Our results are in agreement with the central theme of cellular signaling, wherein cooperation of cellular components results in the coordinated phosphorylation and dephosphorylation of membrane and cytosolic proteins during the transmission of an externally generated signal to the intracellular environment. These externally generated signals by streptococci may occur as a result of the specific interaction of SDH with 30- and 32-kD pharyngeal membrane proteins (Fig. 6), which may in turn be responsible for the observed intracellular signaling events.

The phosphorylation inhibition profile of certain proteins in the presence of genistein and/or staurosporine (Fig. 1 *A*) indicates that SDH is playing a selective role in the initiation of streptococcal-mediated signals in these cells. Thus, the complete inhibition of SDH-mediated phosphorylation of the 17-kD protein by genistein points to the ability of SDH to activate tyrosine kinase. On the other hand, partial inhibition by genistein of whole streptococci–mediated phosphorylation of the 17-kD protein and significant inhibition by a combination of genistein and stauro-

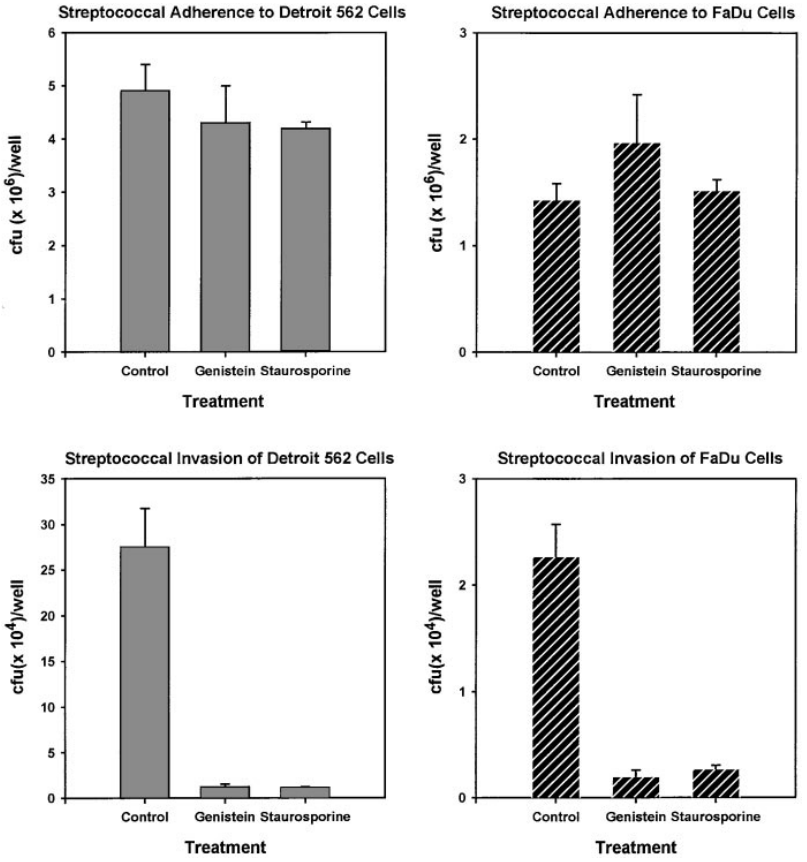


Figure 5. Streptococcal adherence to and invasion of Detroit and FaDu human pharyngeal cells. Role of protein tyrosine kinase and other protein kinases on the streptococcal adherence and invasion was determined by inhibiting these enzymes by specific kinase inhibitors, genistein, and staurosporine. Streptococcal adherence experiments with Detroit cells were carried out for 3 h, while those with FaDu cells were carried out for 1.25 h. The procedures after adhesion assay to determine streptococcal invasion for both the cell types were identical. Number of colonized and invaded streptococci were determined in terms of CFUs. In each experiment, an average of the CFU counts from 4–6 individual wells of 24-well tissue culture plates was calculated. Each *bar* represents the mean value of three such experiments. *Error bars* indicate SD. Genistein or staurosporine has no deleterious effect on the viability of group A streptococci.

sporine, indicates the ability of intact streptococci to induce both serine/threonine kinase(s) and tyrosine kinase. The likelihood of whole streptococci to phosphorylate different amino acid residues within the same protein is also reflected by the fact that interaction of whole streptococci with intact pharyngeal cells leads to higher protein phosphorylation when compared with SDH. We thus speculate that streptococcal surface proteins other than SDH also play a role in initiating signaling pathways.

To understand the role of de novo phosphorylation of the 17-kD protein in the streptococci/SDH-mediated signaling events, we analyzed the NH_2-terminal amino acid sequence of the purified 17-kD protein. The identification of this protein as histone H3 (Fig. 3 *C*) may explain our results regarding streptococcal/SDH-mediated signal transduction. Histone proteins are highly basic molecules found tightly bound to DNA, and are perhaps the best understood structural proteins in the nucleosome. Of the five types of histone proteins (H1, H2A, H2B, H3, and H5), four (other than H1) are called small proteins, which form an octameric complex responsible for DNA coiling in the nucleosome. Histone proteins H3 and H4, which form the inner core of the nucleosome, are the most highly conserved of known histone proteins. Although we have not yet carried out amino acid sequencing of those nonphosphorylated proteins that coprecipitated with the 17-kD protein using antiphosphotyrosine antibodies (Fig. 3 *A*), we anticipate that they are H2A (14.5 kD), H2B (13.8 kD), and H4 (11.4 kD) as revealed from their characteristic migration pattern and reported molecular weight (23, 26, 31). Studies are in progress to verify this suspicion.

We believe that the phosphorylation of histone H3 in the pharyngeal cell nucleus is mediated by a nuclear histone H3–specific kinase that is activated as a result of a series of signal transduction events initiated with the interaction of pharyngeal membrane proteins with streptococci/SDH. To examine whether direct contact of SDH/streptococci with histone proteins is able to induce their phosphorylation, the nuclear fraction of pharyngeal cells was isolated from the M/P fraction by acid extraction (0.3 M HCl and 0.4 H_2SO_4; references 22, 25, 32). The neutralized nuclear extracts containing histone proteins were treated with whole streptococci or purified SDH in the presence of [^{32}P]ATP, as described for other phosphorylation experiments. As we found with the prefractionated M/P fraction (Fig. 1 *B*), the 17-kD protein (histone H3) was not phosphorylated (data not shown), thus confirming that direct interaction of SDH/streptococci with the pharyngeal cell membrane is responsible for the phosphorylation of histone H3.

Signal transduction leading to the phosphorylation of a nuclear protein as a result of an interaction with the host cell surface has not been reported for other bacteria–host systems. Our finding that the phosphorylation of histone H3 is the result of the interaction of streptococci/SDH

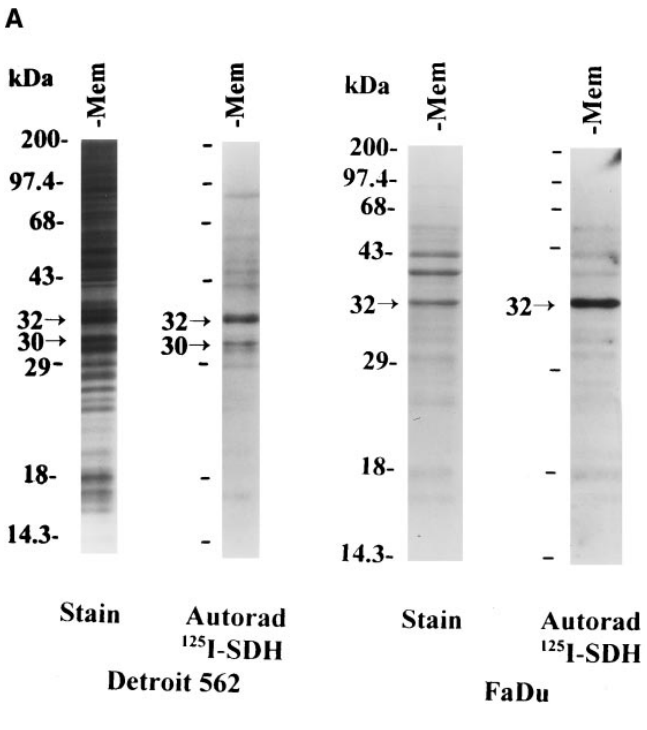


Figure 6. ^{125}I-SDH binding to Detroit and FaDu pharyngeal cell membrane proteins. (*A*) Autoradiograph showing binding of ^{125}I-labeled SDH to pharyngeal membrane proteins (*Mem*). The membrane of Detroit and FaDu cells were separated as described in Materials and Methods. 50 μg of total proteins was resolved on 11% SDS-PAGE, Western blotted on a PVDF membrane, probed with ^{125}I-SDH (1.5×10^5 cpm/ml, sp act: 2×10^6 cpm/μg of SDH), and autoradiographed. A duplicate gel was similarly processed and stained with Coomassie blue. In the membrane fraction of Detroit cells, the major labeled proteins are at 30 and 32 kD (*arrows*), and that in FaDu cells is a 32-kD protein (*arrow*). (*B*) Autoradiograph showing a dose-dependent inhibition of binding of ^{125}I-labeled SDH to the 30/32 kD Detroit pharyngeal membrane proteins in the presence of increasing amounts (5–80 molar excess) of unlabeled purified SDH. PVDF membrane strips containing equal amounts of pharyngeal membrane proteins (100 μg/lane) were probed individually as described in *A* in the presence of increasing amounts of unlabeled SDH in a final volume of 3 ml. The individual strips were then washed, dried, realigned, and autoradiographed for comparison.

with human pharyngeal cells and not other cell lines is in agreement with the tissue tropism of streptococci for these cells in the human host. Phosphorylation of nuclear core proteins including histone H3 is clearly associated with structural changes in chromatin architecture (28). Since phosphorylation of histone H3 correlates directly with a late stage of chromosome condensation in mammalian cells (21–23, 29, 30), streptococcal/SDH-mediated phosphorylation of histone H3 may alter the structure and interaction of nuclear components and influence nucleosome assembly. This possibility is partially supported by our results, which describe ultrastructural changes in pharyngeal cells treated with SDH showing condensed nuclear chromatin (Fig. 3 *B*).

From several published reports, it is now known that the sites of phosphorylation of histone H3 are located near the exposed NH_2-terminus at serine residue 10 or 28 or both (23, 33), and are mediated by Ca^{2+}-dependent H3 kinase (32) or cAMP-dependent protein kinase (30). More recently, chromosome condensation was reported to be associated with enhanced histone H3 phosphorylation mediated by an uncharacterized but staurosporine inhibitable protein kinase (21, 22) or by a mitogen-regulated H3 kinase (34). Interestingly, our results show phosphorylation of histone H3 at both tyrosine and serine, indicating that it is doubly phosphorylated. While serine phosphorylation of histone H3 is well documented, tyrosine phosphorylation has not been previously reported. Because the amino acid sequence of this histone protein is highly conserved from a variety of sources, we speculate that the tyrosines at positions 42, 55, and 100 may be phosphorylated at one or all positions as has been reported for the serine phosphorylation of histone H3 (23, 33). Phosphorylation of pharyngeal histone H3 both at tyrosine and serine suggests that the histone H3-specific kinase may have similarities with some of the MAP kinase family enzymes such as for MEK/JNK/SAPK enzymes, which have been reported to have dual specificities (ser/thr/tyr) for phosphorylation (35, 36). Interestingly, these three enzymes have nuclear proteins as their target molecules. Our ongoing efforts to identify the pharyngeal cell nuclear H3-specific tyrosine kinase and its site of action would not only enable us to understand the role SDH plays in streptococcal pathogenesis, but also to understand the role of tyrosine/serine phosphorylation of histone H3 in chromosomal condensation and its mechanistic link, if any, with gene induction.

Activation of protein tyrosine kinases and other protein kinases including protein kinase C plays a cardinal role in cell morphology, growth, and differentiation (27). In this study, SDH-treated pharyngeal cells have shown significant changes in their shape and size, which were revealed by electron microscopy (Fig. 4). These changes may have biological implications in terms of the ability of pharyngeal cells in presenting an optimum environment for streptococcal adherence and invasion. The fact that genistein and staurosporine significantly inhibit streptococcal invasion (Fig. 5) indicates that group A streptococci may advantageously use SDH and possibly other putative surface components to induce specific protein kinases of pharyngeal cells for successful invasion. From several published reports on host cell interaction with Gram-negative bacteria and listeria (7), it is reasonable to postulate that induction of protein tyrosine kinase and protein kinase C may regulate the activation of Ca^{2+} influx, and increase levels of inositol phosphate (37) and/or diacylglycerol (38) that may in turn facilitate streptococcal invasion through an actin polymerization pathway. Therefore, the ability of streptococci, and SDH in particular, to regulate host signaling events, may have an important impact on the early events of group A streptococcal infection.

In conclusion, the present findings clearly indicate that SDH, both in its purified and cell-bound form, has the ca-

pacity to regulate initial signaling events in pharyngeal cells, and may be one of the first proteins to interact with host cell receptors during streptococcal infection. Once these initial interactions have been established, the host cell may in turn regulate the secretion of certain streptococcal proteins (Pancholi and Fischetti, manuscript in preparation). This cross-talk may be essential in determining the final outcome of disease, and thus is important not only in our understanding of early events in streptococcal infections, but also for other pharyngeal or mucosal infections of bacterial origin, such as those caused by *S. pneumoniae* (12) which also express a surface GAPDH. Similar signaling events may also be envisioned for parasites and fungi reported to express GAPDH as one of their surface molecules (39–43). In addition, we believe that purified streptococcal proteins such as SDH may be used as important biological tools to unravel many of the fundamental aspects of cell biology, such as signaling pathways associated with nucleosomal histone H3-specific kinase, chromatin condensation, and gene induction.

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