Flagellar Adhesion in *Chlamydomonas* Induces Synthesis of Two High Molecular Weight Cell Surface Proteins

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**ABSTRACT** Because our previous studies (Snell, W. J., and W. S. Moore, 1980, *J. Cell Biol.* 84:203-210) on the mating reaction of *Chlamydomonas reinhardtii* showed that there was an adhesion-induced turnover of proteins required for flagellar adhesion, we have used biochemical methods to identify gametic proteins whose synthesis is induced during aggregation. Analysis by SDS PAGE andautoradiography showed that proteins of 220,000 Mr and 165,000 Mr (designated A1 and A2, respectively) consistently showed a high rate of synthesis only in flagella or flagellar membrane-enriched fractions prepared from aggregating gametes. Since the two proteins were soluble in the non-ionic detergent NP-40 and were removed from intact cells by a brief pronase treatment, it is likely that A1 and A2 are membrane proteins exposed on the cell surface.

A1 and A2 were each synthesized by gametes of both mating types (mt- and mt+) and synthesis of these two proteins could be detected in the normal mating reaction (wild type mt— and mt+), in mixtures of mt— and impotent mt+ gametes (which could aggregate but not fuse), and in mixtures of gametes of a single mating type with isolated flagella of the opposite mating type. Cells aggregating in tunicamycin, an inhibitor of protein glycosylation, lost their adhesiveness during aggregation and did not synthesize the 220,000 Mr protein but instead produced a protein (possibly an underglycosylated form of A1) of slightly lower mol wt.

The 220,000 and 165,000 Mr proteins appeared to be flagellar proteins and not cell wall proteins because A1 and A2 did not co-migrate with previously identified cell wall proteins, and synthesis of the two proteins could not be detected in flagella-less (bald-2) mutant cells. Analysis of the adhesive activity of sucrose gradient fraction of detergent (octyl glucoside)-solubilized flagellar membranes revealed that fractions containing A1 and A2 did not have detectable adhesive activity. The possibility remains that A1 and A2 are adhesion molecules whose activity could not be measured in the assay we used. Alternatively, the 220,000 and 165,000 Mr proteins may be inactivated adhesion molecules or else they may be flagellar surface proteins involved only indirectly in the adhesion process.

The mating reaction of the biflagellate alga *Chlamydomonas reinhardtii* begins with flagellar adhesion between gametes of opposite mating types. (See reference 6 for a recent review.) The initial adhesive interaction occurs at random points along the flagellar surfaces, but rather quickly the flagella become aligned tip-to-tip. During this time the ends of the flagella undergo a reaction called flagellar tip activation in which the normally tapered tips of the flagella become elongated and bulbous and material accumulates between the flagellar membrane and the microtubules (12). Possibly as a consequence of flagellar tip activation a signal (probably involving divalent cations [19]) is sent to the cell body, leading to release of a cell wall degrading enzyme(s) (lysin) and activation of actin filament-containing mating structures similar to the acrosomal processes of sperm of many higher organisms (5). In wild-type cells the mating structures fuse, thereby establishing cytoplasmic continuity; finally, the two gametes merge into a quadriflagellated zygote whose flagella are no longer adhesive to each other or to other unmated gametes in the suspension.

We recently reported that aggregating gametes require protein synthesis to maintain their adhesiveness during aggregation (21). Since wild-type mating type minus (mt−) and mating
type pluse (mt') gametes fuse so rapidly after mixing, these experiments were done with impotent (imp) mutants that were able to aggregate with wild-type mt' gametes for several hours or even days, but were unable to fuse. Addition of inhibitors of protein synthesis (cycloheximide or anisomycin) or protein glycosylation (tunicamycin) (17) to mixtures of aggregating mt' and imp mt' gametes led to disaggregation of the cells within 60–90 min. Although Bloodgood (3) has recently reported that vegetative cells show an endogenous turnover of flagellar proteins, with membrane proteins turning over more rapidly than axonemal proteins, we determined that the loss of adhesiveness of the aggregating gametes was a consequence of adhesion, because gametes separately pretreated with cycloheximide for up to 4 h were still fully adhesive.

In other experiments with mt' and imp mt' gametes, we determined that the apparent pool of proteins involved in adhesion began to be depleted within 5 min after gametes were mixed. Within <25 min after mixing, however, replenishment of the apparent pool could be detected and, by 50–55 min after adhesion began, the gametes had acquired their premixing level of proteins involved in adhesion (21). One interpretation of these results was that the cells responded to the loss of proteins involved in adhesion by rapidly synthesizing new molecules. This ability of the cells to respond to loss of a surface function by immediately resynthesizing molecules required for that function was similar to the ability of Chlamydomonas cells to respond to the loss of a flagellum by immediately beginning to synthesize new flagellar proteins (10, 14, 23).

Because these results suggested that the synthesis or glycosylation of flagellar proteins required for adhesion was induced or enhanced by adhesion, we wanted to determine whether any flagellar proteins might show an aggregation-induced increase in synthesis. In the present report we have used electrophoretic methods to analyze flagella and flagellar membrane-enriched fractions prepared from gametes aggregating in the presence of radioactive protein precursors. Parts of this work have appeared in abstract form (20).

MATERIALS AND METHODS

Materials: Pronase, dibucaine, octyl-$\beta$-glucopyranoside (octylglucoside, OG), phenylmethanesulfonyl fluoride (PMSF), cycloheximide, Tris (hydroxymethyl) aminomethane (Tris), sodium dodecyl sulfate (SDS), HEPES, and sucrose were obtained from Sigma Chemical Co. (St. Louis, MO). Tunicamycin was a gift from Dr. Hamill of Eli Lilly. Gel casting materials were obtained from New England Nuclear (Boston, MA). KH$_2$PO$_4$ and K$_2$HPO$_4$ were carrier-free, 43 Ci/mg), $[^{14}H]$arginine, and EN-Hance fluorography reagent were obtained as previously described (16) by transferring vegetative cells (4-7 x 10$^5$) on a 12-h light-dark photoperiod as previously described (16). Gametic cells were isolated from the aggregating gametes by rapidly synthesizing new molecules. This ability of the cells to respond to the loss of proteins involved in adhesion by immediately beginning to synthesize new flagellar proteins (10, 14, 23).

Isolation of Cell Walls: Cell walls were isolated from the medium of aggregating gametes as previously described (18).

Preparation of Conditioned Media: Gametes in LoS N-free medium were concentrated by centrifugation. The two mating types were mixed and, 30 min after centrifugation, the suspension was washed two times at 2,000 $g$ for 4°C. The supernatant was centrifuged again as described above and this supernatant was clarified by centrifugation at 133,000 $g$ (45,000 rpm) for 60 min at 4°C in a Beckman L-50 ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA) fitted with a 50 Ti rotor.

Isotopic Labeling Protocol: Cells (1.6 x 10$^7$ cells/ml) were incubated at room temperature in isotope in 30-, 50-, or 100-ml polystyrene beakers while being stirred at 25–30 rpm with a magnetic stirrer.

Auto-radiography: Dried gels were exposed to Kodak X-Omat X-AR5 film. For fluorography, gels were treated with EN-Hance before drying. ~l-2 x 10$^5$ cpm were loaded in each gel slot, and exposure was made for 24-72 h at -70°C.

RESULTS

To determine the protein labeling pattern of flagella collected from aggregating gametes, we added SO$_4^{2-}$ to a mixture of aggregating mt' and imp mt' gametes 15 min after the gametes had been mixed. After a further 45-min incubation, flagella were isolated from the aggregating cells. Because aggregating cells might release substances into their medium that could affect the specific activity of the radiolabel or alter the metabolic activity of the cells, control, nonaggregating gametes were separately pretreated with conditioned medium from aggregating gametes and then incubated with SO$_4^{2-}$ for 45 min, after which flagella were isolated. The flagella were analyzed by SDS PAGE and autoradiography and the results are shown in Fig. 1. Panel A shows the typical protein staining pattern of aggregating flagella collected from aggregating gametes. Although there were several prominent bands, the identifiable proteins were the major membrane protein (mb), mastigonemes (ms), and the tubulins (tub). Panel B shows autoradiographs of gels of the flagella isolated from mt', imp mt', and the mixed gamete suspensions. Several of the
pattern different from that of the unmixed gametes. Although proteins in the Coomassie Blue-stained gel (panel A) also incorpor

proteins co-migrated with A1 or A2.

proteins in the Coomassie Blue-stained gel (panel A) also incorporated 35SO4 in both nonaggregating and aggregating gametes. The aggregating gametes, however, showed a labeling pattern different from that of the unmixed gametes. Although several proteins sometimes appeared to show increased label-

ing, the proteins of ~220,000 and 165,000 M_r (which we have designated A_1 and A_2) consistently showed a high rate of synthesis in aggregating cells. A_1 and A_2 did not co-migrate with cell wall proteins (panel C) which sometimes contami

nated preparations of flagella. It should be pointed out that, in these and subsequent experiments, we were never able to detect significant amounts of stained protein at the positions of A_1 and A_2. Thus, unless these proteins did not react with Coomassie Blue or the Ag stain, they were of very high specific activity. In addition, we found that the labeling of A_1 and A_2 relative to each other and relative to other flagellar proteins was variable; the cause of this variability is unknown.

During these experiments we noticed that the flagella de-adhered from each other during the deflagellation procedure; possibly due to detergentlike properties of dibucaine, there was also shedding of flagellar membrane vesicles. For these reasons and because it has been reported by several workers that flagella are continuously shedding membrane vesicles (2, 6, 16, 25), we prepared and analyzed three fractions from deflagel-

lated cells: cell bodies, flagella, and a flagellar membrane-enriched fraction. To obtain these fractions, we deflagellated gametes (which in these experiments were aggregating in [3H]arginine) by the dibucaine method, and the cell bodies were sedimented at 2,000 g. The supernatant was then centrifuged at 4,100 g to sediment most of the flagella and the 4,100 g supernatant was centrifuged at 37,000 g to sediment a fraction enriched in membrane vesicles as determined by light and electron microscopy (not shown). These fractions were then analyzed by SDS PAGE and autoradiography. The results (Fig. 2) indicated that although the flagellar fraction (lane e) did contain A_1 and A_2, the flagellar membrane-enriched 37,000 g pellet fraction (lane f) was the fraction most enriched in A_1 and A_2. These proteins were present in very low or undetectable amounts in the deflagellated cell bodies (lane d) (see Fig. 3). As in the experiments with 35SO4, synthesis of A_1 and A_2 was not detected in control, nonaggregating gametes incubated in [3H]arginine (not shown).

Examination of the protein staining pattern (Fig. 2, STAIN) of these [3H]arginine-labeled samples confirmed that the flagellar fraction (lane b) was enriched in tubulin relative to the membrane fraction (lane c) and that the membrane fraction was enriched for the major membrane protein. Although few intact flagella were observed by light microscopy in the 37,000 g pellets, the tubulin may have come from small pieces of flagella still containing axonemes as well as flagellar mem-

brane. In a separate experiment we found that deflagellation was necessary to release A_1 and A_2 from the cells. In contrast to the dibucaine-deflagellated cells, the cells that underwent a mock deflagellation (i.e., cells were subjected to all the steps of the deflagellation procedure except that dibucaine was omitted) still bore flagella and also still contained A_2 (Fig. 3). Although A_1 in the whole cells is obscured by a broad band of slightly more rapidly migrating material, the labeling in the A_1 region is reduced in the dibucaine sample. As expected, the amount of labeling of A_1 and A_2 relative to other cell body proteins was very low because the flagella represent such a small portion (<5%) of the mass of the intact cell.

That A_1 and A_2 were labeled in cells aggregating in [3H]-arginine (Fig. 2) indicated that they were proteins and suggested that the 35SO4 incorporation into A_1 and A_2 in gametes incubated in 35SO4 actually represented new protein synthesis rather than sulfation of pre-existing molecules. Interestingly, in the [3H]arginine-labeled samples, there was no broad band of
FIGURE 2 Analysis of cell bodies, flagella and flagellar membrane-enriched fractions from \[^{[3]}H\]arginine-labeled, deflagellated gametes. Mt- and imp mt\(^+\) gametes (5 ml each, 1.6 x 10\(^7\) cells/ml in LoS N-free medium containing 10 mM HEPES, pH 7.2) were mixed together at \(t = 0\). At \(t = 30\) min, 180 \(\mu\)Ci \[^{[3]}H\]arginine was added and at \(t = 75\) min the cells were washed twice and deflagellated by the dibucaine method. The suspension of deflagellated cells was fractionated as described in Materials and Methods into cell body (CB), flagella (F), and flagellar membrane-enriched (MB) fractions. Ag-stained gels of the samples are shown in the left panel (STAIN) and fluorograms in the right panel (FLUOR). Each lane was loaded with 100,000 cpm. (mb), major membrane protein; (ms), mastigoneme protein; (tub), tubulin, and \(A_1\) and \(A_2\) are indicated by the arrowheads.

labeled material near the top of the gels, although such a band was frequently observed in \(^{35}\)SO\(_4\) labeled samples (Fig. 4). These observations suggested that labeling of this broad band of \(^{35}\)SO\(_4\) material (which was insensitive to pronase [data not shown]) might have been due to the sulfation (24) of pre-existing, non-proteinaceous molecules.

Labeling of \(A_1\) and \(A_2\) in Wild-type Gametes of Both Mating Types

Since the above results were obtained with mixtures of wild-type and mutant cells, we felt it was important to determine whether \(A_1\) and \(A_2\) were detectable in experiments using only wild-type cells. To do this, we incubated aggregating wild-type mt\(^-\) and mt\(^+\) gametes with \(^{35}\)SO\(_4\) at 0-30 min, 30-60 min, and 90-120 min after mixing. The cells were then deflagellated, and membrane-enriched 37,000 g pellets were analyzed as described above. The results shown in Fig. 4A indicated that \(A_1\) and \(A_2\) were synthesized during the first 30 min of aggregation. By 30-60 min, the synthesis of these two proteins was much less and by 90-120 min the amount of \(A_1\) and \(A_2\) being synthesized was extremely low. As the synthesis of these two proteins diminished, there were other unidentified molecules whose synthesis increased during the experiment. A broad band of labeled material near the top portion of the gel appeared to increase during the incubation period, and a protein at about 53,000 \(M_r\) showed an increased rate of labeling. This unidentified 53,000 \(M_r\) protein may be a zygote specific protein described earlier by Weeks and Collis (23).

To determine whether \(A_1\) and \(A_2\) were each synthesized by wild-type gametes of both mating types, we mixed each type of gamete with unlabeled flagella isolated from gametes of the opposite mating type. Fresh flagella were added several times during the entire 4.5-h incubation to keep the cells aggregated, and \(^{35}\)SO\(_4\) was added during the last 30 min of incubation. Flagellar membrane-enriched 37,000 g pellets isolated from the agglutinating gametes were analyzed by SDS PAGE and autoradiography. The results (Fig. 4B) indicated that gametes of both types synthesized \(A_1\) and \(A_2\). The results shown in Figs. 4A and B also indicated that synthesis of \(A_1\) and \(A_2\) was correlated with adhesion. In mixtures of wild-type mt\(^-\) and mt\(^+\) gametes the two proteins were transiently synthesized early...
FIGURE 3 Removal of $A_1$ and $A_2$ from whole cells by dibucaine deflagellation. Mt- and imp mt+ gametes (40 mls each, $1.6 \times 10^7$ cells/ml in LoS N-free medium) were mixed together and allowed to aggregate for 15 min at which time 1.25 mCi $^{35}$SO$_4$ was added. After an additional 75-min incubation the suspension of aggregating cells was collected by centrifugation and washed 2X with 10 mM HEPES (pH 7.2). The sedimented cells were resuspended in 10 ml of HMS-4% and divided into two 5-ml aliquots. One aliquot was subjected to the dibucaine deflagellation procedure and the other aliquot subjected to a mock deflagellation in which distilled water was substituted for dibucaine. Cell bodies or whole cells were then collected by centrifugation at 2,000 g and analyzed by SDS PAGE and autoradiography. CB, cell bodies from dibucaine deflagellation; WC, whole cells from mock deflagellation. The positions of $A_1$ and $A_2$ are indicated by the arrows. Although $A_1$ in the whole cells is obscured by a broad band of slightly more rapidly migrating material, the labeling in this area is reduced in the dibucaine-treated sample. A band migrating in the position of $A_2$ is present in whole cells but absent in the dibucaine-deflagellated sample.

in the mating reaction when aggregation was maximum (Fig. 4A). As the flagella became nonadhesive when the gametes had fused to form zygotes, synthesis of the two proteins diminished. However, in flagella-gamete mixtures, the two proteins were still synthesized by these aggregating cells 4 h after aggregation was initiated.

Effects of Pronase and Tunicamycin on $A_1$ and $A_2$

To determine whether the two proteins were on the surface of the cells, we incubated mt- and imp mt+ gametes with $^{35}$SO$_4$ for 30 min during aggregation. The gametes were then incubated with 0.1 mg/ml of pronase for 7 min at room temperature until the cells had disaggregated. Under these conditions, except for the loss of adhesiveness, the cells appeared by light microscopy to be unaffected by the enzyme treatment. Control cells were treated in the same way except that no pronase was added. At the end of the incubation, the cells were washed twice, deflagellated, and the membrane-enriched 37,000 g pellet fractions were analyzed by SDS PAGE and autoradiography. The wash solutions and deflagellation solutions contained 2 mM PMSF. The results (Fig. 5) indicated that, whereas the control, non-pronase-treated sample showed substantial labeling of $A_1$ and $A_2$ (Fig. 5, $^{35}$SO$_4$, cn), the pronase-treated sample (Fig. 5, $^{35}$SO$_4$, pro) had little if any $A_1$ or $A_2$. The protein staining patterns (PROTEIN) of the pronase-treated (pro) and control (cn) samples were nearly identical; this demonstrated that tubulin, an internal flagellar protein, was unaffected by the pronase treatment and thus confirmed the light microscopic observation that the flagella remained intact during the pronase treatment. Moreover, the major membrane protein, mastigome protein, and most of the other proteins were also unaffected by the brief pronase treatment. In other experiments we
and imp mt⁺ gametes lost their adhesiveness 90–120 min after addition of tunicamycin to the medium of aggregating cells, whereas nontreated gametes continued to aggregate. Since this suggested that tunicamycin-sensitive glycoproteins might be involved in the maintenance of adhesiveness during adhesion, we wanted to determine whether A₁ and A₂ would be synthesized in cells aggregating in tunicamycin. To do this, we mixed gametes together in the presence of 1 μg/ml tunicamycin and after 15 min added 35SO₄ for 45 min. The cells were then deflagellated and the membrane-enriched fraction was analyzed by SDS PAGE and autoradiography. The results (Fig. 5, 35SO₄, tum) indicated that, although a protein of the original mobility of A₁ was not synthesized by the gametes aggregating in tunicamycin, a new protein of slightly lower molecular weight, possibly an under-glycosylated form of A₁, appeared in the gels of the tunicamycin-treated samples. The experiments illustrated in Fig. 5 suggested that A₁ was a flagellar surface glycoprotein. Other experiments (not shown) revealed that A₁ and A₂ were soluble in the non-ionic detergent NP-40; this result was also consistent with the idea that the two proteins were membrane proteins.

Sucrose Gradient Analysis of A₁ and A₂

The results presented above indicate that synthesis of the 220,000 and 165,000 Mr proteins was induced by adhesion and suggested that the proteins might be directly involved in the adhesive interaction. To determine whether A₁ and A₂ might have adhesive activity, we fractionated detergent (OG)-solubilized, membrane-enriched fractions from aggregating gametes on OG-containing sucrose gradients, and the gradient fractions were then tested for adhesive activity and analyzed for the presence of the two proteins by SDS PAGE and autoradiography. Fig. 6 shows the autoradiographs of the gels of the gradient fractions. A₁ and A₂ appeared approximately one-quarter of the way down the gradient. Qualitative determinations of the adhesive activity by use of the assay described by Adair et al. (1) of fractions from such gradients revealed that the peak of adhesive activity was in the fraction indicated by the arrow; fractions containing A₁ and A₂ showed no detectable adhesive activity. Thus, these experiments indicated that A₁ and A₂ did not cofractionate with adhesive activity detectable by this assay.

Comparison to Cell Wall Proteins

It was also possible that A₁ and A₂ were cell wall proteins whose synthesis was induced by the cell wall loss that occurs during adhesion. Although it has been shown that cells lose their walls during gamete-gamete and gamete-flagella interactions (6, 8, 18), to our knowledge there is no information in the literature on re-synthesis of wall proteins. Comparison of the SDS PAGE pattern of flagellar membrane-enriched fractions from aggregating cells to that of isolated cell walls indicated that the membrane-enriched fractions sometimes contained labeled proteins that comigrated with proteins found in isolated cell walls (see below). A₁ and A₂, however, did not co-migrate with previously identified wall proteins (Fig. 1 C).

To further investigate the possibility that A₁ and A₂ might be wall proteins present in low amounts whose synthesis was induced by adhesion, we incubated separately vegetative (non-adhesive) and gametic (adhesive) cells with conditioned medium prepared from aggregating gametes. We and others have previously shown that such conditioned medium contains cell
cells had lost their walls, $^{35}$SO$_4$ was added to the suspension in wall degrading (lysin) activity (6, 8, 18). After 90-100% of the deflagellated and the membrane-enriched 37,000 g pellets an-
same amount of time. After 45 rain of labeling, the cells were
the 35SO$_4$ was added. As a control, gametes not incubated with
portions of the treated gametic cells were mixed together before
the continued presence of the conditioned medium. Equal
could be detected in the lysin-treated gametic preparations,
amounts of labeled material that co-migrated with A1 and A2
alyzed by SDS PAGE and autoradiography (Fig. 7).
detected in nonaggregating cells. A further analysis of some of
ative cells or in the non-lysin-treated gametic cells. The results
and less or none at all was detected in the lysin-treated vege-
tative proteins in the aggregating ceil sample. Additional
experiments are needed before concluding that there was an
actual suppression of synthesis of wall proteins during aggre-
gation.
As a further means of testing the idea that A$_1$ and A$_2$ might
be wall proteins, flagella-less mutant cells (bald-2 cells) incu-
bated in N-free medium in continuous light overnight to induce
gametogenesis were treated with conditioned medium until
wall loss was 80-100% complete. These cells were then incub-
bated in $^{35}$SO$_4$ and, after 30 min, the cells were treated as if
they had flagella and carried through the deflagellation pro-
cedure. Analysis of the nearly undetectable 37,000 g pellets by
SDS PAGE and autoradiography (Fig. 8) indicated that there
was synthesis of cell wall proteins, but no labeled material
migrating in the region of A$_1$ and A$_2$ could be detected in these
37,000 g pellets or in the cell bodies (not shown).

**DISCUSSION**

The results presented in this report indicate that flagellar
adhesion during the initial stages of the mating reaction of
Chlamydomonas reinhardtii was accompanied by a dramatic
increase in the synthesis of two proteins that co-purified with
a flagellar membrane-enriched fraction prepared from deflag-
gellated, aggregating gametes. These two promise-sensitive, labeled
dspecies did not co-migrate with previously identified
Chlamydomonas flagellar proteins, nor were significant
amounts of protein co-migrating with these two radiolabeled
bands detected by Coomassie Blue or silver staining of the gels.
In addition, A$_1$ and A$_2$ were not labeled in nonaggregating or
flagella-less cells.

The role of the two proteins in the mating reaction is as yet
undetermined, but synthesis of A$_1$ and A$_2$ was induced early in
the mating reaction and appeared to be shut down after the
gametes fused to form zygotes. It seems likely, therefore, that
A$_1$ and A$_2$ are important in the initial stages of aggregation. By
creating conditions in which cells agglutinated but were unable
to fuse (i.e., by adding isolated gametic flagella to gametes of
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FIGURE 7 Effect of conditioned medium on synthesis of A1 and A2 by vegetative and gametic cells. Vegetative mt⁻ and imp mt⁺ cells (5 ml each, 1.1 x 10⁷ cells/ml in LoS Medium I containing 10 mM HEPES, pH 7.2) were incubated with 2 ml of conditioned medium collected from 1.1 x 10⁹ aggregating gametes. After 34 min, a quantitative assay for wall loss (18) revealed that 80-100% of the cells had lost their walls. The cells were then incubated for 46 min in 3 mCi ³⁵SO₄, and flagellar membrane-enriched fractions were collected and analyzed as described. A similar procedure was used to obtain flagellar membrane-enriched fractions from mt⁻ (10 ml), imp mt⁺ (5 ml) and aggregating mt⁻ and imp mt⁺ (5 ml each, mixed together in the presence of conditioned medium 30 min before the 45-min incubation in 3 mCi ³⁵SO₄). Control mt⁻ and imp mt⁺ gametes were not incubated with conditioned medium. The brackets at the top of the gel indicate control (cont.) or conditioned medium (cond. med.)-treated samples and the brackets at the bottom indicate samples from vegetative (veg) or gametic (gam) cells. Major membrane (mb), mastigoneme (ms), tubulin (tub), A1 and A2 and three cell wall proteins (cw) (see Fig. 1 C) are indicated by the arrowheads. The + and − symbols indicate the mating type(s) of the cells from which the samples were prepared.

the opposite mating type or by mixing mt⁻ and imp mt⁺ gametes), we induced the synthesis of the two proteins and maintained it for several hours. In mixtures of wild-type mt⁻ and mt⁺ gametes, however, which fused to form zygotes within

FIGURE 8 Absence of synthesis of A₁ and A₂ in bald-2, flagella-less cells. Vegetatively growing mt⁻ and mt⁺ bald-2 cells were induced to undergo gametogenesis by transferring the cells into LoS N-free medium containing 10 mM HEPES, pH 7.2. After 18 h in continuous light, 10 ml of each cell type and a mixture of 5 ml of each type (1.6 x 10⁷ cells/ml) were incubated with conditioned medium prepared from 1.6 x 10⁹ aggregating mt⁻ and imp mt⁺ gametes. After 30 min, when 80-100% of the cells had lost their walls, 3 mCi ³⁵SO₄ was added to each sample and incubation continued for a further 45 min. As expected, none of the cells had flagella and no aggregation was observed in the mixture of bald-2 cells of opposite mating types. At the end of the labeling period the samples were subjected to the dibucaine deflagellation method and “membrane-enriched fractions” were prepared and analyzed by SDS PAGE and autoradiography (bald). Control membrane-enriched fractions prepared from aggregating mt⁻ and imp mt⁺ gametes are shown for comparison (cont.). Cell wall protein (cw), tubulin (tub), and A₁ and A₂ are indicated by the arrowheads.

60 min after mixing, A₁ and A₂ were synthesized only during the first 30–60 min after mixing, and by 90 min their synthesis was very low. This pattern of labeling is distinctly different from that observed by other investigators who have studied synthesis of zygote proteins. We have found that synthesis of A₁ and A₂ is rapidly curtailed after gametic fusion, whereas synthesis of proteins described by Minami and Goodenough (13) and Weeks and Collis (23) was induced by fusion.

Although it appeared that the increased synthesis of these
two proteins was induced by flagellar adhesion, it is not yet clear whether flagellar adhesion per se or events signalled by flagellar adhesion led to synthesis of A1 and A2. Our report that aggregating gametes required protein synthesis to maintain their adhesiveness (21) suggested that proteins directly involved with adhesion were being consumed and must be continuously resynthesized during adhesion; A1 and A2 are candidates for these proteins. The pronase sensitivity of the labeled 220,000 and 165,000 Mr proteins in intact, aggregating cells is consistent with the surface localization of putative adhesion molecules. Moreover, the electrophoretic mobility of A1 was found to be altered when aggregating mt- and imp mt+ gametes were incubated during the labeling period in tunicamycin, a glycosylation inhibitor that we have previously shown caused loss of flagellar adhesiveness. One interpretation of these latter results is that in the presence of tunicamycin the molecules were synthesized but not fully glycosylated and would thus show an altered electrophoretic mobility and would not be adhesive.

On the other hand, fractionation studies on flagellar membrane-enriched samples solubilized in octyl glucoside showed that A1 and A2 did not co-purify on sucrose gradients with adhesive activity. The absence of adhesive activity in the A1- and A2-containing fractions could mean that these two proteins were not involved in adhesion. Possibly, however, A1 and A2 were adhesion molecules whose adhesive properties could not be detected with the assay that we used, or A1 and A2 may have been degradation products of adhesion molecules. In this regard, we determined that A1 and A2 did not co-migrate with previously identified (4) proteolytic degradation products of the major membrane protein (data not shown).

Another possibility is that A1 and A2 were involved in the formation and maintenance of activated flagellar tips. As Mesland et al. (12) and we (17) have shown, flagellar tips remain in the activated state as long as flagellar adhesion persists; as cells de-adhere, the tips return to the unactivated state. Proteins necessary for tip activation may also undergo rapid synthesis and turnover during adhesion; thus, even though they would not themselves be adhesive, they would show increased incorporation of radioactivity only during adhesion.

Finally, it may be that A1 and A2 are cell wall proteins whose synthesis was induced by the wall loss that normally occurs during the mating reaction. We feel, however, that it is unlikely that A1 and A2 are cell wall proteins, because flagella-less mutant cells treated as if they were wild-type cells and incubated in condition medium did not synthesize A1 and A2 even though wall protein synthesis did occur. Nevertheless, we have shown (Fig. 7) that gametes that were de-walled by incubation in lysin-containing medium showed 35SO4 incorporation into wall proteins. That these labeled wall proteins were in the 37,000 g pellet fraction indicated that the flagellar membrane-enriched fraction was contaminated with nonflagellar components. In addition, in some experiments, small amounts of synthesis of A1 and A2 could be detected in gametes separately incubated in conditioned medium. Because the conditioned medium also contained agglutinin activity, however, we are at present unable to determine whether it was the agglutinin activity or the lysin in the conditioned medium that sometimes led to synthesis of A1 and A2 in preparations of gametes of a single mating type. It is also possible that wall loss itself is an integral part of the signaling mechanism and not only induces new wall synthesis, but also leads to or permits subsequent steps requiring protein synthesis in the mating process.

In conclusion, our previous work (17, 21) indicated that flagellar adhesion was accompanied by an adhesion-induced turnover of flagellar proteins required for adhesion. In the present studies we have identified two cell surface proteins that are candidates for these adhesion-induced molecules. To determine the role of A1 and A2 in the mating reaction will require their further purification and precise determination of the cellular localization of these two proteins.

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