Flagellar Adhesion between Mating Type Plus and Mating Type 
Minus Gametes Activates a Flagellar Protein-tyrosine Kinase during 
Fertilization in Chlamydomonas

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When Chlamydomonas gametes of opposite mating type are mixed together, flagellar adhesion through sex-specific adhesion molecules triggers a transient elevation of intracellular cAMP, leading to gamete activation in preparation for cell-cell fusion and zygote formation. Here, we have identified a protein-tyrosine kinase (PTK) activity that is stimulated by flagellar adhesion. We determined that the protein-tyrosine kinase inhibitor genistein inhibited fertilization, and that fertilization was rescued by dibutyryl cAMP, indicating that the genistein-sensitive step was upstream of the increase in cAMP. Incubation with ATP of flagella isolated from non-adhering and adhering gametes followed by SDS-PAGE and immunoblotting with anti-phosphotyrosine antibodies showed that adhesion activated a flagellar PTK that phosphorylated a 105-kDa flagellar protein. Assays using an exogenous protein-tyrosine kinase substrate confirmed that the activated PTK could be detected only in flagella isolated from adhering gametes. Our results indicate that stimulation of the PTK is a very early event during fertilization. Activation of the PTK was blocked when gametes underwent flagellar adhesion in the presence of the protein kinase inhibitor staurosporine, but not in the presence of the cyclic nucleotide-dependent protein kinase inhibitor, H8, which (unlike staurosporine) does not block the increases in cAMP. In addition, incubation of gametes of a single mating type in dibutyryl cAMP failed to activate the PTK. Finally, flagella adhesion between plus and minus fla10-1 gametes, which have a temperature-sensitive lesion in the microtubule motor protein kinesin-II, failed to activate the PTK at elevated temperatures. Our results show that kinesin-II is essential for coupling flagellar adhesion to activation of a flagellar PTK and cAMP generation during fertilization in Chlamydomonas.

When mating type plus (mt+) and mating type minus (mt−) Chlamydomonas gametes are mixed together, they adhere to each other via sex-specific adhesion molecules, the mt+ and mt− agglutinins, on their flagella surfaces and then undergo a series of events composing gamete activation that prepares the gametes for cell fusion. Gamete activation includes cell wall loss, movement of proteins (including agglutinins) from the cell body to the flagella, modification of the flagellar tip, phosphorylation of a gamete-specific homeodomain protein involved in activation of zygote-specific genes (1), activation of mating structures, and redistribution of the adhesion/fusion protein Fus1 onto the surface of the mt+ mating structure (Ref. 2, reviewed in Ref. 3).

Several years ago it was shown that one of the earliest biochemical events triggered by flagellar adhesion is a 10–20-fold increase in intracellular cAMP (4, 5) brought about by adhesion-induced activation of a flagellar adenylyl cyclase (6–9). Related experiments showing that addition of dibutyryl cAMP to gametes of a single mating type induced all of the events known to occur during flagellar adhesion has confirmed that cAMP is a key second messenger in Chlamydomonas fertilization (3, 5). Consistent with a central role for cAMP in gamete activation, H8, an inhibitor of cAMP-dependent protein kinase A does not interfere with flagellar adhesion (5) or the adhesion-induced rise in intracellular cAMP (9), but all of the downstream events that normally accompany the increase in cAMP are blocked by H8.

In previous studies we have established that the protein kinase inhibitor staurosporine blocked flagellar adhesion-induced activation of the gamete-specific flagellar adenylyl cyclase (7–9). More recently, we determined that the microtubule motor protein, kinesin-II, also is essential for the flagellar adhesion-induced increase in cAMP (10). We found that fla10-1 gametes, which have a mutation in the kinesin-II gene (11–13), exhibited wild type levels of flagellar adhesion, but failed to undergo gamete activation (10, 14). Our studies showed that the fla10 gametes were blocked at the step that couples agglutinin interactions to activation of adenylyl cyclase.

Here, we have investigated the very early events that occur following flagellar adhesion to identify a flagellar protein kinase activity stimulated by adhesion, and we examined the role of kinesin-II in protein kinase activation. We show that incubation of gametes in the protein-tyrosine kinase inhibitor, genistein, does not interfere with flagellar adhesion, but inhibits the gamete activation that follows as assessed by gamete fusion. The block can be overcome by incubation of the gametes in dibutyryl cAMP, implicating a protein-tyrosine kinase in the pathway that connects flagellar adhesion to activation of adenylyl cyclase. Biochemical experiments demonstrate that immediately after flagellar adhesion is initiated, a flagellar protein-tyrosine kinase (PTK) is activated whose substrate is a soluble, ~105-kDa flagellar protein. Our data indicate that flagellar adhesion per se, and not the downstream events, in-
including protein kinase A activation and gamete fusion that normally accompany flagellar adhesion, is sufficient to activate the PTK. Moreover, microtubule-based motility and intraflagellar transport are connected to regulation of the PTK, because the PTK is not activated during flagellar adhesion between fla10-I mt+ and mt− gametes, which have a lesion in the microtubule motor protein, kinesin-II.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Anti-phosphotyrosine antibody (anti-Tyr(P)) 4G10 was obtained from Upstate Biotechnology (Lake Placid, NY), HEPES, N-[2-(methylamino)ethyl]-5-isouquinoline sulfonamide (H8), staurosporine, dibutyryl cyclic AMP (Bt2-cAMP), cAMP, ATP, papaverine, and genistein were obtained from Sigma. All other chemicals were reagent grade. The ECL Western blotting kit was from Amersharm Biosciences.

Cells and Cell Culture—Chlamydomonas reinhardtii strains 21gr (mt+) (CC-1690), 6145c (mt−) (CC-1158) (all available from the Chlamydomonas Genetics Center, Duke University), and strains fla10-I (4930 3–4, mt+) and fla10-I (4930 6–2, mt−) (provided by Dr. Susan Dutcher, Washington University, St. Louis) were cultured vegetatively in liquid culture with aeration and gametic cells were obtained as described previously (15). To activate gametes of a single mating type, cells were incubated with 15 mM Bt2-cAMP and 150 μM papaverine in N-free medium for ~30 min. Assessments of gamete activation as determined by assaying cell wall loss and zygote formation as determined by formation of quadriflagellated cells were carried out as described previously (10). For the experiments at 32°C, wild-type gametes and fla10-I mutant gametes were incubated with illumination in a water bath with constant aeration.

Isolation of Flagella—Flagella were isolated by use of the pH shock method of Witman et al. (16) with minor modifications as follows. Gametes (typically ~6 liters, at 5 × 107 cells per ml in N-free medium) were harvested by centrifugation at 3,000 × g for 3 min and resuspended in 5% sucrose in 20 mM HEPES, pH 7.2. The cells were placed on ice and the flagella were immediately detached by rapidly reducing the pH to 4.5 using 0.5 M acetic acid with constant stirring. Deflagellation, which usually occurred within 15 s, was confirmed using phase-contrast microscopy and the cells were neutralized with 0.5 M KOH. Cell bodies were removed by centrifugation through a 15-ml cushion of 25% sucrose in 20 mM HEPES, pH 7.2, in 50-ml conical tubes at 3,000 × g for 9 min. The remaining cell bodies were removed from the top layer by centrifugation through a second 25% sucrose, 20 mM HEPES layer. Flagella were harvested from the overlying supernatant by centrifugation in an HB-4 rotor at 8,000 rpm for 15 min. The resulting pellet was thoroughly resuspended to a concentration of ~3 mg/ml protein in 5% sucrose, 20 mM HEPES, pH 7.2, containing a 1/10 dilution of the protease inhibitor mixture for plant cells from Sigma (catalogue number P9599). The suspension was divided into small portions, frozen in liquid nitrogen, and stored at ~70°C.

For fractionation, flagella (~300 μg of protein in 100 μl 5% sucrose,
The results were because of activation of a PTK or, for example, a change in accessibility of the substrate, we used an exogenous protein-tyrosine kinase substrate, polyglutamine tyrosine (4:1) (PGT) to assay for an adhering-gamete enzyme that phosphorylates a 105-kDa flagellar protein was activated soon after flagellar adhesion-induced gamete activation. The control, non-adhering mt- and mt- gametes that had been mixed together for 10 min (mt+ & mt-), were incubated as above in the protein kinase assay buffer for the indicated times followed by SDS-PAGE and immunoblotting with anti-Tyr(P). Phosphorylated 105-kDa protein was not detected in any of the samples at 0 min and did not appear at any time in the flagellar samples from the mt+ or mt- gametes alone. In the flagellar samples isolated from adhering mt+ and mt- gametes (mt+ & mt-) phosphorylated 105-kDa protein was detectable within 2 min after the incubation with ATP began and continued to increase during the 15-min assay.

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sucrose, 20 mM HEPES was added to a final concentration of 5% sucrose and the whole flagellar samples were assayed for PTK activity using the "Experimental Procedures" and the whole flagellar samples were assayed for PTK activity using the endogenous substrate in the in vitro assay as described above. B, flagella (100 μl containing ~300 μg of protein in 5% sucrose, 10 mM HEPES, pH 7.2) isolated from adhering gametes were frozen and thawed and centrifuged to obtain the freeze/thaw soluble fraction. The sedimented flagella were resuspended in 100 μl of buffer A, which contains 0.1% Nonidet P-40, for 10 min on ice, and centrifuged again to yield the 0.1% Nonidet P-40-soluble and -insoluble fractions. The Nonidet P-40 insoluble fraction was resuspended in buffer A, and equal volumes (5 μl) of the starting material and each fraction were incubated for 10 min in PTK buffer and assayed for PTK activity as above.

We investigated the time during fertilization when the PTK was activated. To do this, mt+ and mt− gametes were mixed together and at various times after mixing, the flagella were detached and isolated and samples were assayed for the presence of the active PTK. As shown in Fig. 4A while PTK activity was high in flagella isolated from gametes that had been mixed for 3 min or more, the PTK activity was detectable in flagella isolated from gametes that had been mixed for only 30 s. These results indicated that PTK activation took place very soon after gametes adhered to each other, most likely before gamete fusion, for example, which begins to take place 3–5 min after mixing. To further examine the properties of the PTK and its substrate, we identified the flagellar compartment in which the PTK and the substrate were localized. For fractionation, the flagella were disrupted by freezing and thawing and the sample was centrifuged to yield the freeze/thaw supernatant (matrix fraction). The sedimented flagella with their disrupted membranes (20, 21) were further fractionated by detergent extraction and centrifugation and equivalent portions of each fraction were assayed for PTK activity. As shown in Fig. 4B, phosphorylation of the 105-kDa protein was detected only in the starting sample of whole flagella and in the initial freeze/thaw supernatant from the disrupted flagella. The results indicated that both the protein kinase and its substrate were released from flagella by freezing and thawing.

Activation of the Flagellar PTK Occurs at a Very Early Step during Gamete Interactions—We used the protein kinase inhibitors staurosporine and H8 to examine the stage during flagellar adhesion and gamete activation that the PTK was activated. Previously, we showed that while flagellar adhesion was unaffected by these inhibitors, gamete activation was blocked, albeit at different steps by each inhibitor. The staurosporine-sensitive block is upstream of activation of adenylyl cyclase, as cAMP levels fail to increase when gametes undergo flagellar adhesion in the presence of staurosporine (9). We have also shown that H8, an inhibitor of cyclic nucleotide-dependent protein kinases, does not block the flagellar adhesion-induced increase in cAMP, but inhibits steps in gamete activation, including cell wall release and cell-cell fusion, that are downstream of activation of adenylyl cyclase (9). To test the effects of the inhibitors on activation of the PTK, we preincubated mt+ and mt− gametes in each inhibitor and mixed the cells together in the continued presence of the inhibitors. Examination by light microscopy indicated that, as previously reported (9), flagellar adhesion occurred normally between mt+ and mt− gametes in the presence of each of the inhibitors, but cell fusion was blocked (not shown). Flagella were isolated from mt+ and mt− gametes that were mixed together in the presence and absence of each of the inhibitors as well as from control, non-treated, adhering cells and assayed for the PTK activity. As shown in Fig. 5A, the PTK became activated in flagella of mt+ gametes alone (mt+, mt−), and from control, adhering mt+ and mt− gametes that had been mixed together for 3 min (mt+ & mt−) also are shown. Right panel, flagella isolated from mt+ gametes alone and from mt− gametes alone were analyzed for activation of PTK in PTK assays that included 100 μM cAMP. As shown in Fig. 5A, the PTK became activated in flagella of mt+ gametes alone (mt+, mt−), and from control, adhering mt+ and mt− gametes that had been mixed together for 3 min (mt+ & mt−) also are shown. Right panel, flagella isolated from mt+ gametes alone and from mt− gametes alone were analyzed for activation of PTK in PTK assays that included 100 μM cAMP. Activated previously, we showed that while flagellar adhesion was unaffected by these inhibitors, gamete activation was blocked, albeit at different steps by each inhibitor. The staurosporine-sensitive block is upstream of activation of adenylyl cyclase, as cAMP levels fail to increase when gametes undergo flagellar adhesion in the presence of staurosporine (9). We have also shown that H8, an inhibitor of cyclic nucleotide-dependent protein kinases, does not block the flagellar adhesion-induced increase in cAMP, but inhibits steps in gamete activation, including cell wall release and cell-cell fusion, that are downstream of activation of adenylyl cyclase (9). To test the effects of the inhibitors on activation of the PTK, we preincubated mt+ and mt− gametes in each inhibitor and mixed the cells together in the continued presence of the inhibitors. Examination by light microscopy indicated that, as previously reported (9), flagellar adhesion occurred normally between mt+ and mt− gametes in the presence of each of the inhibitors, but cell fusion was blocked (not shown). Flagella were isolated from mt+ and mt− gametes that were mixed together in the presence and absence of each of the inhibitors as well as from control, non-treated, adhering cells and assayed for the PTK activity. As shown in Fig. 5A, the PTK became activated in flagella of mt+ gametes alone, adhering cells (C) and in flagella isolated from gametes adhering in the presence of H8, whereas activation of the PTK did not occur in flagella isolated from gametes that had undergone flagellar adhesion in the presence of staurosporine (St). These results indicated that activation of the PTK required a staurosporine-sensitive step and suggested that the PTK was activated at an early stage during gamete activation, downstream of agglutinin interactions and flagellar adhesion and upstream of the appearance of cAMP.
Fig. 6. Activation of the adhesion-induced PTK fails in fla10-1 gametes. Wild type mt+ and mt− gametes or kinesin-II mutant fla10-1 mt+ and fla10-1 mt− gametes were incubated for 40 min at 21 or 32 °C prior to mixing of the respective cells at the same temperature as the pretreatment. The fla10-1 gametes mixed together at 32 °C showed maximal agglutination at 10 min, after which time their flagella were isolated. Flagella were isolated from the other 3 samples 3 min after mixing. 10 µl of the freeze/thaw supernatant fractions from the flagella was assayed for PTK activity (upper panel). The lower panel shows the same membrane stained for SksC, a 48-kDa Chlamydomonas protein, as a loading control.

Fig. 7. Model of signal transduction pathway activated by flagellar adhesion between mt+ and mt− gametes. Agglutinin-mediated flagellar adhesion between mt+ and mt− gametes leads to activation of a staurosporine-sensitive serine-threonine protein kinase(s), which in turn activates a genistein-sensitive PTK whose substrate is the 105-kDa protein. The kinesin-II sensitivity of PTK activation indicates that the motor protein functions very early in the pathway. Subsequent to phosphorylation of the 105-kDa protein, adenyl cyclase is activated, and the consequent increase in cAMP leads to gamete activation and fusion to form a quadriflagellated zygote.

The PTK Is Not Activated during Flagellar Adhesion in fla10-1 Gametes, Which Contain a Lesion in the Microtubule Motor Protein Kinesin-II—Previously, we showed that coupling of flagellar adhesion to the increase in cAMP was disrupted in gametes of the fla10-1 mutant (10, 14), which express a kinesin-II with a temperature-sensitive defect in the 90-kDa motor subunit FLA10 because of a single amino acid substitution in the motor domain (13). The defective kinesin-II is expressed in fla10-1 gametes, but at much lower levels than in wild type cells. When fla10-1 cells are grown at room temperature they are fully flagellated and exhibit IFT. On the other hand when grown at elevated temperature (32 °C), the cells do not have flagella (13, 21, 24). In cells grown at room temperature and shifted to 32 °C, IFT particle movement ceases after about 40 min and within 1–2 h, the flagella begin to resorb (12, 21, 24). For our experiments, fla10-1 mt+ and fla10-1 mt− gametes cultured at 21 °C were shifted to 32 °C for 40 min, mixed together, and their flagella were isolated 10 min after mixing. Flagella also were isolated from adhering mt+ fla10-1 gametes and mt− fla10-1 gametes that were mixed together at 21 °C, from wild type gametes mixed together at 21 °C, and from wild gametes shifted to 32 °C for 40 min before being mixed together for 3 min. As shown in Fig. 6, when analyzed by the in vitro assay, flagellar samples from wild type gametes mixed at 32 °C showed a slight reduction in ability to phosphorylate the 105-kDa protein compared with flagellar samples isolated from wild type gametes mixed at 21 °C. On the other hand, although phosphorylation of the 105-kDa protein was similar to wild type levels in assays of the 21 °C fla10-1 samples, phosphorylated 105-kDa protein was not detected in assays of the 32 °C fla10-1 samples. The bottom panel of Fig. 6, which is a loading control, shows the same samples probed with an antibody against a 48-kDa Chlamydomonas protein (25, 26), and documents equivalent protein loading. Thus, coupled with their failure to undergo increases in cAMP during flagellar adhesion at elevated temperature (10), fla10-1 gametes also failed to show flagellar adhesion-induced activation of the PTK.

DISCUSSION

Previous studies on the molecular events regulated by flagellar adhesion during fertilization in Chlamydomonas indicated that flagellar protein kinases are activated at an early step after adhesion between mt+ and mt− flagella, before the adhesion-induced increase in cAMP (7–9, 20, 26). In addition, we recently reported that in cells with a defect in the microtubule
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motor protein kinesin-II, gamete activation was blocked at a step downstream of flagellar adhesion but upstream of the adhesion-induced increase in cAMP (10). Here, we identified a protein-tyrosine kinase activity that was regulated by flagellar adhesion per se, and not by the downstream events that accompany flagellar adhesion such as increased levels of cAMP, activation of cyclic nucleotide-dependent protein kinases, or cell fusion. Initial experiments with the PTK inhibitor genistein implicated PTK activity in fertilization downstream of flagellar adhesion and upstream of the increase in cAMP (Fig. 1). Use of in vitro PTK assays revealed a PTK activity that was present in flagella isolated from adhering gametes and that was nearly undetectable in flagella isolated from gametes of either mating type that had not been mixed with gametes of the opposite mating type (and therefore had not undergone flagellar adhesion). Importantly, flagellar adhesion was sufficient to activate the PTK, but not in gametes with a lesion in the microtubule motor protein, kinesin-II. A working model consistent with these data is that flagellar adhesion leads to a rapid stimulation of PTK activity in a pathway involving kinesin-II-dependent intraflagellar transport. PTK activation is followed, in turn, by activation of the adenyl cyclase and gamete activation (Fig. 7).

For detection of the activated PTK in these experiments, it was essential to incubate the flagellar samples with ATP before carrying out SDS-PAGE and immunoblotting. As shown in Fig. 2, when ATP was left out of the in vitro assay, phosphorylated 105-kDa protein was not detected; similarly, in the experiments to examine the relationship between the extent of phosphorylation and assay time, no phosphorylation of the 105-kDa protein was detected in the 0 time point sample. Previous experiments from our laboratory and others (7, 9, 26, 27) on protein kinase activity in Chlamydomonas have shown that flagella contain high levels of protein phosphatase activity, and the omission of phosphatase inhibitors in the in vitro assay used in the current experiments led to much reduced levels of phosphorylation (not shown). Use of the exogenous PTK substrate PGT was important as an independent confirmation of PTK activation and also should be useful for future experiments to identify and further characterize this adhesion-regulated PTK.

Several independent approaches indicated that activation of the PTK was a very early event during gamete activation: activation required flagellar adhesion, occurred within seconds after gametes of opposite mating type were mixed together, and took place at the same time or earlier than the increases in levels of cAMP shown previously (5, 10). The results strongly indicated that PTK activation was not dependent on increases in cAMP or cell fusion and was induced by flagellar adhesion per se. Thus, fus1-1 mutant gametes, which are capable of flagellar adhesion but not gamete fusion, showed adhesion-dependent activation of the PTK. Similarly, when gametes underwent flagellar adhesion in the presence of the inhibitor of cyclic nucleotide-dependent protein kinases, H8, gamete activation was blocked as assessed by the absence of cell wall loss and gamete fusion, but the PTK still was activated. And, while incubation of gametes of a single mating type in dibutyryl cAMP bypasses the requirement for flagellar adhesion and induces the cellular changes that normally accompany flagellar adhesion, the PTK was not activated in such artificially activated cells. In addition, inclusion of cAMP in the in vitro assay for PTK activity in flagella isolated from gametes of a single mating type did not lead to activation of the PTK. Whereas the evidence is compelling that PTK activation is not downstream of activation of adenyl cyclase and that regulation of both activities is tightly coupled to flagellar adhesion, determining whether PTK activation is upstream of activation of the flagellar adenyl cyclase or in a parallel signaling pathway remains to be achieved.

Activation of the PTK (Fig. 4) and increases in cAMP (5, 9, 10) are the earliest biochemical or cellular events that have been demonstrated after flagellar adhesion is initiated, occurring within seconds after cells are mixed together. Activation of the PTK (Fig. 5) and the increase in cAMP and gamete activation are blocked when cells undergo flagellar adhesion in staurosporine (9), suggesting that the activity of one or more protein kinases is essential for activation of the PTK and the adenyl cyclase. Finally, adhesion-induced stimulation of the PTK (Fig. 6), increases in cAMP and gamete activation (10) fail in fla10-1 gametes, which have a lesion in the microtubule motor protein kinesin-II (13). The simplest model that explains the data is that a single signaling pathway is initiated by flagellar adhesion. Because cAMP-regulated events are downstream of the PTK, it is likely that the PTK is upstream of adenyl cyclase. In addition, all of the cellular events characterized to date that compose gamete activation, including flagellar tip movement, movement of agglutinin molecules from the cell body to the flagella, cell wall loss, and activation of mating structures are downstream of activation of adenyl cyclase. That is, all of these cell surface responses can be induced by incubation of gametes of a single mating type in dibutyryl cAMP. Therefore, if the PTK is part of a signaling pathway that is independent of cAMP, the events induced by that putative pathway have not yet been reported.

The observation that fla10-1 gametes at elevated temperature fail to activate the PTK (Fig. 6) is the second demonstration that kinesin-II plays a direct or indirect role in adhesion-induced signaling in Chlamydomonas (10, 28). Microtubule motor proteins have been implicated in other signal transduction pathways in Chlamydomonas (10, 28) (reviewed in Refs. 3, 29, and 30) and it will be interesting to determine the role of kinesin-II function in coupling agglutinin interactions during flagellar adhesion to gamete activation. For example, kinesin-II-dependent movement of IFT particles might be required for maintaining proper levels of key signaling proteins in the flagella. Or, binding between mt+ and mt− agglutinin molecules might trigger conformational changes in the interacting molecules that induce them to associate with kinesin-II or IFT particles as an early step in signal transduction. The results presented here should now make it possible to continue a detailed dissection of the molecular pathways that underlie cell-cell adhesion-induced signal transduction during fertilization in Chlamydomonas.

Acknowledgements—We thank Dr. Susan Dutcher (Washington University, St. Louis, MO) for providing the fla10-1 mutant strains. We also thank our colleagues Drs. Fred Grinnell and Melanie Cobb for helpful discussions.

REFERENCES

1. Zhao, H., Lu, M., Singh, R., and Snell, W. J. (2001) Genes Dev. 15, 2767–2777
2. Misamore, M. J., Gupta, S., and Snell, W. J. (2003) Mol. Biol. Cell. 14, 2530–2542
3. Pan, J., and Snell, W. J. (2000) Curr. Opin. Microbiol. 3, 596–602
4. Pijut, H. L. A., van Driel, R., Janssen, P. M. W., Musgrave, A., and van den Ende, H. (1984) FEBS Lett. 174, 132–136
5. Pasquale, S. M., and Goodenough, U. W. (1987) J. Cell Biol. 105, 2279–2292
6. Saito, T., Small, L., and Goodenough, U. W. (1993) J. Cell Biol. 122, 137–147
7. Zhang, Y. H., Ross, E. M., and Snell, W. J. (1991) J. Biol. Chem. 266, 22654–22659
8. Zhang, Y., and Snell, W. J. (1993) J. Biol. Chem. 268, 1786–1791
9. Zhang, Y., and Snell, W. J. (1994) J. Cell Biol. 125, 617–624
10. Pan, J., and Snell, W. J. (2002) Mol. Biol. Cell 13, 1417–1426
11. Huang, B., Rifkin, M. R., and Luck, D. J. (1977) J. Cell Biol. 72, 67–85
12. Lux, F. G. D., and Dutcher, S. K. (1991) Genetics 128, 549–561
13. Waltzer, Z., Vashishthia, M., and Hall, J. L. (1994) J. Cell Biol. 126, 175–188
14. Piperno, G., Mead, K., and Henderson, S. (1996) J. Cell Biol. 133, 371–379
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15. Snell, W. J. (1976) *J. Cell Biol.* **68**, 48–69
16. Witman, G. B., Carlson, K., Berliner, J., and Rosenbaum, J. L. (1972) *J. Cell Biol.* **54**, 507–539
17. Wilson, N. F., O’Connell, J. S., Lu, M., and Snell, W. J. (1999) *J. Biol. Chem.* **274**, 34838–34848
18. Allis, C. D., Chicoine, L. G., Glover, C. V., White, E. M., and Gorovsky, M. A. (1988) *Anal. Biochem.* **159**, 58–66
19. Hulsen, D., Baron, A., Salisbury, J., and Clarke, M. (1991) *Cell Motil. Cytoskeleton* **18**, 113–122
20. Zhang, Y., and Snell, W. J. (1995) *Methods Cell Biol.* **47**, 459–465
21. Cole, D. G., Diener, D. R., Himmelhau, A. L., Beech, P. L., Fuster, J. C., and Rosenbaum, J. L. (1998) *J. Cell Biol.* **141**, 993–1008
22. Goodenough, U. W., Hwang, C., and Martin, H. (1976) *Genetics* **82**, 169–186
23. Goodenough, U., Armbrust, E., Campbell, A., and Ferris, P. (1995) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **46**, 21–44
24. Kozminski, K. G., Beech, P. L., and Rosenbaum, J. L. (1995) *J. Cell Biol.* **131**, 1517–1527
25. Kurvari, V., Zhang, Y. H., Luo, Y. X., and Snell, W. J. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 39–43
26. Zhang, Y., Luo, Y., Emmett, K., and Snell, W. J. (1996) *Mol. Biol. Cell* **7**, 513–527
27. Bloodgood, R. A. (1992) *Exp. Cell Res.* **198**, 228–236
28. Pan, J., and Snell, W. J. (2003) *J. Cell Sci.* **116**, 2179–2186
29. Pan, J., Missmore, M. J., Wang, W., and Snell, W. J. (2003) *Traffic* **4**, 452–459
30. Rosenbaum, J. L., and Witman, G. B. (2002) *Nat. Rev. Mol. Cell Biol.* **3**, 813–825