*Proc. Natl. Acad. Sci. USA*
Vol. 94, pp. 11767–11769, October 1997

# Commentary

# Phosphotyrosine signaling and the single cell:metazoan boundary

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This commentary is prompted by two recent articles concerning slime molds (Mycetozoa)—one in this issue of the *Proceedings* by Sandra Baldauf and W. Ford Doolittle entitled "The origin and evolution of the slime molds (Mycetozoa)" (1) and a recent article by Jeffrey Williams and colleagues entitled "SH2 signaling in a lower eukaryote: A STAT protein that regulates stalk cell differentiation in dictyostelium" (2). Together these articles provoke thought about the origin of phosphotyrosine in cell signaling and its possible importance in slime molds, or some similar organism, in crossing the single cell–metazoan evolutionary boundary.

Systematists classify together three groups of organisms that are referred to as "slime molds," the Myxogastria, Dictyostelia, and Protostelia (3). The Protostelids are the least studied. The Myxogastria include the well studied *Physarum polycephalum*, a prototypical single-celled ameba that can also form a large multinucleate plasmodium (4). *Dictyostelium discoideum*, described in 1935 by K. B. Raper (5) and studied intensively for almost 50 years by developmental biologists (6, 7), is the classic Dictyostelid. All three groups share the important characteristic features of both growing as single cells and differentiating into multicellular, stalked structures called fruiting bodies that bear haploid spores in a spore cup.

The Baldauf and Doolittle (1) paper provides a molecular phylogenetic review and a comparison of new sequences of several slime mold translation elongation factor 1$\alpha$ (EF1$\alpha$) proteins that together lead to a strong conclusion: the Mycetozoa are of monophyletic origin and are more closely related to fungi and animals than to plants. The paper by Williams and colleagues (2) follows the tradition of using *Dictyostelium discoideum* for developmental studies. This organism grows as individual ameboid cells until starved, when in response to secreted cAMP (8) the cells aggregate into a large mound. Aggregation is followed by a highly orchestrated series of events that offer the chance to study the molecular basis for cell determination (6, 7). The mound of aggregated cells rises into a tubular structure that falls over and becomes a "slug" that is capable of migration. Cells on the anterior end of the slug differentiate into stalk cells (prestalk and then stalk cell steps can be distinguished). The posterior cells are pushed up through the stalk and become spore cells held in the spore cup. Many genes are known to be differentially expressed in time and in cell type during this developmental progression (7–9). Williams and colleagues (2) have studied the regulation of genes encoding extracellular matrix proteins that are expressed in anterior cells (10–12). They have found the evolutionarily earliest, presently known use of phosphotyrosine -SH2 signaling and shown the importance of this pathway in cell determination.

Phosphotyrosine signaling, first recognized more than 20 years ago in mammalian cells in response to growth factors, is widely believed to be a key signal in cell determination and differentiation in vertebrates and invertebrates (13). To some considerable surprise the *Dictyostelim discoideum* signaling molecule employing phosphotyrosine signaling is not a member of the receptor tyrosine kinase family, e.g., like the EGF, PDGF, or insulin receptors, nor is it a member of the nonreceptor tyrosine kinase family, like the src protein and its many relatives (14). Rather it is a STAT-like protein. The STATs are a more recently recognized family of molecules that are latent transcription factors, which, in mammals, are activated by phosphorylation on a single tyrosine subsequent to polypeptide binding to the cell surface receptors (15). The activated STATs then translocate to the nucleus to participate in gene activation. These results fuel speculation, originating with that by Tony Hunter (see pp. 923–924 in ref. 13), the discoverer of phosphotyrosine in proteins, that I will discuss and defend; namely, that tyrosine phosphorylation was crucial for metazoan evolution. Further, it may be that the new discovery of Williams and colleagues (2) shows us which SH2 phosphotyrosine signaling molecules came first. Finally, that this discovery occurred in *Dictyostelium* may inform us about the boundary in evolution between single cells and metazoans.

**The Slime Molds Form a Clade.** The major issue addressed by Baldauf and Doolittle (1) is the relatedness of the three groups of Mycetozoa to each other and their close relation to the fungal–animal branch of the eukaryotic "crown" of organisms. Comparisons of the sequences of the small subunits of ribosomal RNA in different organisms have been invaluable as a first step in molecular phylogenetic comparisons (16). For example, this type of comparison established the three lineages of all cells on this planet, the Archaea, Eucarya, and Bacteria. Also, ribosomal RNA comparisons showed that among the multicellular groups of organisms in the eukaryotic crown, fungi and animals are more closely related to each other than either is to plants (17). Baldauf and Palmer (18) subsequently used comparisons of protein sequences to strongly substantiate the fungal/animal similarity. Baldauf and Doolittle (1) point out that although ribosomal RNA comparisons do not place the slime molds together phylogenetically, such comparisons may not be a correct means of comparing these organisms. For example, the ribosomal RNA sequences of Mycetozoa vary from among the lowest to the highest GC content (42.5 to 52.9%) of all eukaryotic ribosomal sequences. Furthermore, the small rRNA sequence of *Physarum* appears on several counts to be rapidly changing. Baldauf and Doolittle (1) therefore turn to protein sequencing comparisons to relate the Mycetozoa to one another and to other organisms.

Earlier analyses of actin sequences showed Myxogastrids and Dictyostelids to be highly similar (19), although their ribosomal RNAs were not closely similar. Comparison of proteins in the translation apparatus, obviously one of the early evolutionary requirements of cells, is being used to great advantage in assaying evolutionary relatedness (1). EF1$\alpha$ is prominent in such work, and Baldauf and Doolittle report new sequence data on this protein from each of the three classes of Mycetozoa. Careful analysis of these new sequences by both parsimony and boot strap techniques shows unambiguously that the three groups of Mycetozoa form a clade (a monophyletic lineage). Furthermore, comparison with other organisms shows the Mycetozoa to belong in the crown of eukaryotes along with other multicellular lineages. This conclusion is in accord with earlier, less sophisticated sequence comparisons of

a group of about 20 proteins from humans, yeast, and *Dictyostelium* (20, 21). This early work did not include sufficiently widespread comparisons between organisms to be generally compelling but is in accord with the results of Baldauf and Doolittle (1).

**Dictyostelium Has a Stat Molecule.** Large-scale direct sequence comparison on complete genomes is the inexorable, definitive method for determining the path of evolution. Pending the availability of such sufficient complete sequences, comparisons of key regulatory proteins among a broad set of selected organisms can be very informative. It is in this context that the study of Williams and colleagues (2) promises to have a profound impact on evolutionary studies.

Williams and his group, among others, have studied genes that are activated by signals that arise during development and differentiation of *Dictyostelium discoideum* (7–12). As noted above, the anterior cells in the *Dictyostelium* slug differentiate into stalk cells under the influence of a secreted, chlorinated hexaphenone called DIF (22). Genes that are induced at specific times and sites in prestalk cells have been defined. Genomic clones of these induced genes have been examined, and the regulatory DNA sequences have been mapped by transgenic analysis in the whole organism. Proteins from induced cells that bind to the prestalk cell regulatory elements were identified (12). When the DNA-binding protein was purified and the gene was cloned and sequenced, a surprise was in store (2). By sequence comparison, the regulatory protein showed a similarity to the mammalian STAT proteins in two key regions. First, the GTFLLRFSE sequence, which embraces the key arginine (R) for phosphotyrosine binding in the STAT -SH2 group and which is almost completely conserved in mammals and *Drosophila* (23), appears as GTFIIRFSE in the *Dictyostelium* protein. In addition, in the DNA-binding region ≈150–175 amino acids upstream of the SH2 region, mammalian proteins have another highly conserved sequence, SLPVVV/II (24). Mutations in the valines of this region block DNA binding (24). In the *Dictyostelium discoideum* protein there is a sequence PFPVVI about 100 aa upstream of the presumed SH2 boundary. Perhaps most convincingly, the purified DNA-binding version of the *Dictyostelium* protein contains phosphotyrosine, and the phosphotyrosine is integral to DNA binding. The protein binds DNA as a dimer, and this binding is prevented by a phosphopeptide embracing a single tyrosine that lies just downstream of the presumptive SH2 region—the same position as the single tyrosine that is phosphorylated in all the mammalian STATs. This same assay originally was used to interrupt tyrosine-phosphorylated Stat1 dimers (25), and this phosphopeptide interruption of various SH2 phosphotyrosine interactions has been widely used. Thus, it appears that Williams and colleagues (2) have isolated a developmentally important *Dictyostelium discoideum* protein, and the protein is a STAT-like molecule that becomes tyrosine phosphorylated, dimerizes, and binds DNA. Of interest is the lack of a -COOH domain in the *Dictyostelium* protein (the presumptive phosphotyrosine is only 7 aa from the -COOH terminus). This is the region where the transactivation domain of mammalian STATs is located (1). Although the importance of DIF as an activator of anterior cell development is established (12, 22), what triggers phosphorylation of the *Dictyostelium* Stat is not clear. *Dictyostelium* is not known to have receptor tyrosine kinases or nonreceptor tyrosine kinases such as the src-like kinases (14). Tyrosine kinases (26, 27) and phosphotyrosine phosphatases (28–30) have been described in *Dictyostelium*, but their function is not yet clear. At least one of these kinases is a dual (serine and tyrosine) specific kinase that is active in microfilament contraction (26). Another is recently described by Adler *et al.* (27) and has the interesting property of having two distinct but incomplete catalytic domains similar to the design of the Jak kinases.

**Fitting the New Findings into the Evolutionary Occurrence of Phosphotyrosine Signaling.** The discovery of phosphotyrosine in animals cells was followed by the description of several types of tyrosine kinases, notably, receptor tyrosine kinases such as epidermal growth factor, platelet-derived growth factor, insulin receptor, etc., and nonreceptor tyrosine kinases such as those of the src family (ref. 13; see also ref. 14). Studies of many animals have shown that invertebrates down the phylogenetic scale to Cnidarians (coelenterates such as hydra) and sponges contain both receptor tyrosine kinases and src-like proteins (31). However, the recently completed *Saccharomyces cerevisiae* sequence (32) shows no evidence for tyrosine kinases of this type in *Saccharomyces*. There are tyrosine kinases in yeast, such as those in the mitogen-activated protein kinase pathway (14), but they lack the easily recognizable tyrosine kinase domain seen in receptors and src-like proteins. Plants likewise are not known to contain receptor tyrosine kinases or nonreceptor (src-like) tyrosine kinases. Sequence data (obtained through expression-tagged sequence analysis) on two fungi, *Aspergillus nidulans* and *Neurospora crassa*, are estimated to be approximately 50% complete. In neither database is there a recognizable receptor or src-like tyrosine kinase at the present time (C. M. Horvath and J.E.D., Jr., unpublished observations). Against this background, the discovery of a SH2/phosphotyrosine-containing molecule in a potential precursor to metazoans—if slime molds can be considered such—is of considerable interest. Further, the *Dictyostelium discoideum* Stat has a role in anterior cell determination and differentiation and is required for cell growth (J. G. Williams, personal communication). These are the pervasive roles of -SH2 phosphotyrosine signaling in animals (see pp. 923–924 in ref. 13).

In considering precursors to multicellular organisms it is widely supposed that the chlorophyta (chloroplast containing single-celled organisms) are the precursors to plants (33). Because of the recognition of the close amino acid sequence similarities that link fungi and animals (17, 18), a common, single-celled precursor to fungi and animals might be entertained. However, any precursor to animals might logically be expected to engage in phosphotyrosine signaling. Finding a phosphotyrosine signaling molecule in an organism capable of growing as a single cell and also of multicellular development greatly heightens the evolutionary interest of the discovery of Williams and coworkers (2). From the single but significant difference of EF1-$\alpha$ sequences between three different slime molds and all of the fungi and animals (12 missing amino acids in slime molds that are present in all fungi and animals examined; see ref. 18), Baldauf and Doolittle (1) conclude that present-day slime molds should not be considered directly in the line to metazoans. Nevertheless, occurrence of an SH2/phosphotyrosine in *Dictyostelium* surely should stimulate searches among other single-celled organisms, particularly those that have the capacity to become multicellular, for evidence of phosphotyrosine -SH2 signaling, because this signaling mechanism is used heavily to control growth and differentiation in metazoans.

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