expect that cells in the suspensions synchronize, owing to their coupling through extracellular cAMP. Much less is known about the dynamic behavior of coupled chaotic systems. If individual cells were evolving on the same strange – i.e., chaotic – attractor, at different phases (owing to different initial conditions), or on different strange attractors, their coupling might well result in the transformation of aperiodic into regular oscillations. Such a possibility is currently under investigation, by means of numerical simulations with the model for periodic and aperiodic cAMP signalling [7, 8, 11]. If it turns out that a population of individually chaotic cells shows global periodic behavior as a result of strong intercellular coupling, one might expect that experiments in suspensions would not yield results similar to those obtained in the experiments in which the amoebae were aggregating on agar [5] and were therefore not subjected to the strong coupling that occurs in cell suspensions.

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Endocytosis and inositol hexakisphosphate levels in *ras* transformants of *Dictyostelium discoideum* amoebae [1]

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Summary. Fluid-phase pinocytosis kinetics and lysosomal enzyme secretion parameters were measured in *Dictyostelium discoideum* amoebae constructed from strain AX3 by transformation with a multicopy plasmid carrying either a normal *ras* gene (*ras*-Gly12), a mutated *ras* gene (*ras*-Thr12) or by the vector carrying the geneticin resistance gene only (pDNEO2). It was found that the pinocytosis rate and extent as well as the lysosomal enzyme secretion were slightly different in the three strains. These changes, however, were related to minor modifications of the cellular volumes. The overall concentration of inositol hexakisphosphate was similar in the three strains.
Key words. Fluid-phase pinocytosis; enzyme secretion; inositol hexakisphosphate; *ras* genes; *Dictyostelium discoideum*; amoebae.

The protein products of the *ras* proto-oncogenes (p21 *ras*) bind GTP and show a restricted homology with signal-transducing guanine nucleotide binding proteins (G-proteins). Point mutations at specific positions in the *ras* genes have been associated with phenotypic transformation leading to increased malignancy [2–4]. In the slime mold *Dictyostelium discoideum*, a *ras* gene (Dd *ras*) is developmentally regulated and seems to be essential for growth [5–7]. The introduction of a missense mutation at the amino acid 12 induced phenotypic changes and perturbations in the formation of aggregation centers during the early chemotactic phase [8, 9]. Recent studies suggest that *Dictyostalium ras* is involved in the inositol-lipids pathway of the signalling system [10].

Microinjection of human Ha-*ras* protein in rat embryo fibroblasts has been shown to enhance membrane ruffling and fluid-phase pinocytosis of fluorescein labelled dextran (FITC-dextran) by a factor of ten [11]. Similarly, measurements of Lucifer Yellow pinocytosis in a *Saccharomyces cerevisiae* strain mutated at the level of its RAS2 gene (analogous to the Ki-*ras*) have revealed a higher level of endocytosis than in the isogenic strain with the normal *ras* gene [12]. Uptake of horse radish peroxidase was found to be increased by a factor of two in NIH/3T3

cells transfected with the human Ha-*ras* Val12 oncogene[13]. In contrast to these results, the *ras* protein has been shown not to be associated with exocytosis processes[14].

We have examined the fluid-phase pinocytic capacity and lysosomal enzyme secretion of *Dictyostelium* strains transfected with multiple copies of either the normal *ras* gene (*ras*-Gly12) or a mutated form (*ras*-Thr12). The results show no significant differences in endocytosis in contrast to the previously cited reports in other cellular systems.

Both in mammals and *Dictyostelium, ras* seems to affect inositol 1,4,5 triphosphate (IP3) level[10]. In *Dictyostelium*, after labelling with [^{3}H]inositol, radioactivity in an HPLC peak corresponding to inositol hexakisphosphate (IP6) was also increased when mutated *ras* genes were present[10]. Since this compound can be detected by ^{31}P NMR, we decided to analyze it further, as well as the major phosphorylated metabolites of the different *Dictyostelium ras* strains. The results show no significant differences in the basal inositol hexakisphosphate level between these strains. The apparent contradiction between these data and labelling experiments will be discussed.

Materials and methods. Cell culture. *Dictyostelium discoideum* strains used in this work were three transformants of AX3 (ATCC 28368), pDNEO2[24], *ras*-Gly12 and *ras*-Thr12 constructed as described previously[8]. They were grown at 22 °C in axenic medium[15] supplemented with 25 µg/ml geneticin. Cells in their logarithmic phase of growth were collected for experiments.

Mean cell volume. Cell number and size distribution were determined with a Coulter counter model Z4 coupled to a multichannel pulse height analyzer (Channelyzer 256). Cell counts were accumulated over 64 channels from 0 to 1730 µm^3. The mean cell volume, Vm was calculated directly from the individual channel data (i = 1–64): $Vm = \Sigma (Ni \times Vi)/\Sigma Ni$, Ni and Vi being respectively the number and volume of cells in a given channel.

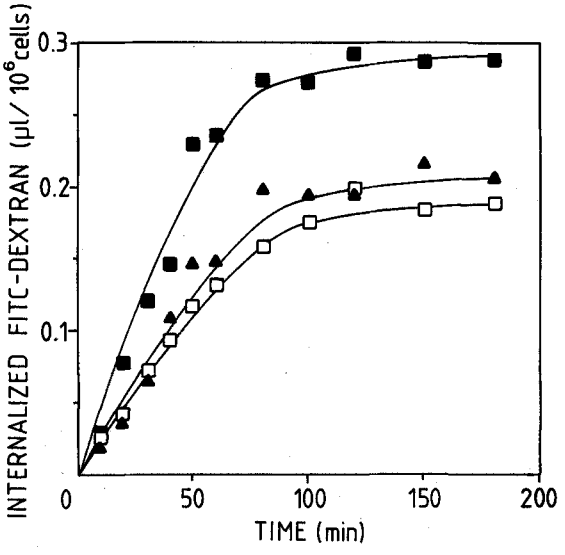
Fluid-phase pinocytosis. Pinocytosis kinetics were measured in 40 mM Mes-Na buffer, pH 5.3 at 22 °C with FITC-dextran as a fluid-phase marker[16, 17]. Uptake was expressed as an endocytic index[18], defined as the volume of incubation medium whose content has been captured by a given number of cells in a given time (µm^3/cell × min).

Lysosomal enzyme secretion. Sucrose-stimulated secretion of acid phosphatase was carried out as described[19].

^{31}P NMR of $HClO_4$ extracts of aerobic amoebae. ^{31}P NMR was performed as described previously[21, 22].

Chemicals. FITC-dextran (FD 70, Mr = 70 000) was purchased from Sigma and geneticin (G 418) from Gibco.

Results and discussion. Fluid-phase pinocytosis of FITC-dextran in 40 mM Mes-Na buffer, pH 5.3, was measured in the two *ras* transformants, *ras*-Gly12 and *ras*-Thr12, as well as in a control transformant, pDNEO2[24], which contains uniquely the selectable neo^R gene (fig.). Kinetic curves in all cases were characterized by a linear phase of FITC-dextran uptake lasting for about 90 min until a plateau was reached. The plateau was previously shown to correspond to a strict dynamic equilibrium between fluid entry and evacuation by pulse experiments[17]. When examined on a per cell basis and when the pinocytosis parameters were expressed as fractional values of cell volume, fluid-phase pinocytosis activity was quite similar in the three cell types (table 1). By statistical analysis, we can show that the difference between the three strains is not significant. This is in clear contrast to the situation reported in fibroblasts, where microinjection of Ha-*ras* protein greatly stimulated fluid-phase pinocytosis[11]. Stimulation of pinocytosis in yeast was not quantified[12]. Several hypotheses can be put forward to explain this different behavior. For example, in *Dictyostelium* amoebae which rely entirely on a very active fluid-phase pinocytosis to fulfil their nutritive needs during growth in axenic medium, the endocytic machinery could well already be at maximum capacity and no longer under any *ras* control. Alternatively, endocytosis could be modulated only through a specific member of the *ras* gene family, present in fibroblasts, 3T3 cells or yeast, which have more than one *ras* gene, but absent in *Dictyostelium* which possesses a single *ras* gene.



Fluid-phase pinocytosis of FITC-dextran by *Dictyostelium* amoebae from strains pDNEO2, *ras*-Gly12 and *ras*-Thr12. Amoebae were suspended at 5 × 10^6 cells/ml in 40 mM Mes-Na buffer pH 5.3 at 22 °C. At t = 0, FITC-dextran was added at a final concentration of 2 mg/ml and 1-ml aliquots were taken at indicated times, quenched in 10 ml ice-cold 17.5 mM KPi buffer, 0.05% (w/v) bovine serum albumin, pH 6.0. After centrifugation at 1200 x g for 4 min at 0–4 °C, cells were washed twice in 10 ml of the same buffer. Cells were resuspended in 1 ml ice-cold KPi buffer and a 50-µl fraction was counted. The amount of internalized FITC-dextran was then measured by fluorometry (λ_{ex} = 470 nm, λ_{em} = 520 nm) in the remaining volume (0.95 ml) after addition of 2 ml 50 mM Na_2HPO_4, 0.25% (w/v) Triton X-100. Internalized FITC-dextran is represented as a function of time. (□): pDNEO2; (■): *ras*-Gly12; (▲): *ras*-Thr12.

Table 1. Kinetics parameters of fluid-phase pinocytosis in *Dictyostelium* transformants

	pDNEO2	*ras*-Gly12	*ras*-Thr12
Mean cell volume (Vm) (μm^3/cell)	685 ± 26	872 ± 146	810 ± 122
Endocytic index			
(μm^3/min × cell)	3.0 ± 0.6	4.6 ± 0.4	3.8 ± 0.9
(% of Vm/min)	0.44 ± 0.1	0.53 ± 0.07	0.47 ± 0.07
Endocytosis level			
(μm^3/cell)	172 ± 22	262 ± 54	220 ± 34
(% of Vm)	25 ± 3	30 ± 3	27 ± 1

Values are the mean ± SEM of four independent experiments.

Secretion of lysosomal enzymes is another facet of endocytosis-linked membrane flow. It can be considerably stimulated in *Dictyostelium* amoebae upon addition of non-metabolizable sugars[19]. Sucrose-stimulated secretion of acid phosphatase was superimposable (not shown) in the three strains, pDNEO2, *ras*-Gly12 and *ras*-Thr12, in agreement with a previous report indicating that the *ras* protein in adrenal medullary cells was not part of the machinery involved in exocytosis[14].

N-*ras* p21 has been suggested to be a putative Gp protein in the inositol phospholipid pathway[20]. A recent report showed, after incubation of *Dictyostelium* with [^{3}H]inositol, a 3–5-fold rise in the levels of radioactivity incorporated into inositol 1,4,5 triphosphate (IP3) and inositol hexakisphosphate (IP6) in *ras*-Thr12 strain compared to *ras*-Gly12 strain[10]. The authors proposed that the *Dictyostelium ras* gene product participates in the inositol phosphate signal transduction pathway. IP6 concentrations were thus measured by ^{31}P NMR in perchloric acid extracts of pDNEO2, *ras*-Gly12 and *ras*-Thr12 cells, obtained in optimized conditions to avoid any loss of polyphosphorylated compounds[21, 22]. Results for IP6 and some other compounds are summarized in table 2. The higher Pi level seen in the cells containing extra copies of the non-mutated *ras* gene could reflect an increased phosphate turnover rate. The significance of this result remains unclear.

Nucleoside di- and triphosphates (NDP, NTP) and phosphomonoester levels are alike in the different strains. IP6 level is also not influenced by the presence of a mutated *ras* gene. This result seems to contradict earlier studies using labelled inositol precursors. In those experiments the cells were incubated in non-nutrient medium (phosphate buffer) so that the labelled compound could penetrate the cell without being diluted. Our cells may not have been under identical metabolic conditions. Another possible explanation would be that the introduction of a mutated *ras* gene has no direct effect on the basal level of IP6, but that it increases the metabolic turnover rate of both IP3 and IP6 by a factor of 3 to 5. In this respect, it is worth realizing that the intracellular IP6 concentration in *Dictyostelium* amoebae is in the half millimolar range. IP6 may thus possibly be a storage compound in *Dictyostelium*, and *ras* could influence its storage or its mobilization rate.

Table 2. Concentrations of Pi, NDP, NTP, IP6 and other phosphomonoesters in *Dictyostelium* transformants

	pDNEO2	*ras*-Gly12	*ras*-Thr12
Pi	2.5	3.5	2.6
NDP	0.18	0.20	0.16
NTP	1.2	1.2	1.2
IP6	0.46	0.49	0.43
Other phosphomonoesters	1.4	1.3	1.3

Pi concentration (mM) in the extracts was determined by the method of Fiske and SubbaRow[23]. Concentrations (mM) of the other compounds was calculated from the area of their peak relative to the Pi peak in the ^{31}P NMR spectra of the perchloric acid extracts[22].

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