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Production and activity of spore differentiation factors (SDFs) in *Dictyostelium*

Christophe Anjard[1], Wen Tsan Chang[2], Julian Gross[2] and Wolfgang Nellen[1,*]

[1]Universität Kassel, Abt. Genetik, Heinrich-Plett-Strasse 40, D-34 132 Kassel, Germany
[2]University of Oxford, South Park Road, Oxford OX1 3 QU, UK
*Author for correspondence (e-mail: nellen@hrz.uni-kassel.de)

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SUMMARY

SDF-1 and SDF-2 are peptides that promote terminal spore differentiation under submerged conditions. The present study shows that they accumulate differentially and are released during the development of wild-type cells and can promote spore formation in cells disaggregated from wild-type culminants. SDF-1 accumulates during the slug stage and is released in a single burst at the onset of culmination while SDF-2 accumulates during early culmination and is released in a single burst from mid-culminants. The effects of SDF-1 and SDF-2 on stalk cell formation in cell monolayers were investigated. SDF-1 by itself induces stalk cell formation in some strains and also synergizes with the stalk-cell-inducing factor, DIF-1. cAMP has an inhibitory effect on stalk cell formation when either DIF-1 or SDF-1 are present on their own but is almost not inhibitory when both are present. SDF-2 alone does not induce stalk cell formation and appears to inhibit the response to DIF-1. At the same time, it increases the extent of vacuolization of the stalk cells that are produced. We propose that the release of SDF-1 and then of SDF-2 may mark irreversible steps in the developmental programme associated, respectively, with culmination and spore maturation.

Key words: *Dictyostelium*, Differentiation, SDF, Spore Differentiation Factor, Culmination

INTRODUCTION

In *Dictyostelium*, as in other eukaryotes, cellular differentiation is a multistep process. While the initial differentiation steps are reversible, later stages are irreversible and lead to terminal differentiation and morphogenesis. *Dictyostelium* differentiation is initiated by starvation. Individual cells aggregate and eventually form a multicellular organism containing up to about 10^5 cells comprising two main cell types: stalk cells and spores (reviewed in Loomis, 1996). Pulsatile emission of cAMP organizes chemotactic streaming towards aggregation centres and induces aggregation-specific genes. A morphological tip forms on the aggregates, which elongate to form an upright column of cells that may collapse onto the substratum to form a migrating slug. Up to the slug stage, cells rapidly de-differentiate when disaggregated in the presence of a food source (Finney et al., 1987; Takeuchi and Sakai, 1971). Also, if the prespore and prestalk zones forming a slug are mechanically separated, the pattern reforms by re-differentiation of cells (Bühl and MacWilliams, 1991; Lokeshwar and Nanjundiah, 1983).

Culmination is triggered by environmental conditions such as desiccation or overhead light (Gross, 1994). Slugs stop migrating and reorganize morphologically while their component cells undergo terminal differentiation (Dormann et al., 1996). Prestalk cells differentiate by entering into a stalk tube to which they contribute by releasing cellulose and matrix proteins. They finally vacuolize and expand, forming a growing stalk. Prespore cells differentiate into spores as they are lifted up by the stalk. A spatial gradient of expression of spore-specific genes has been observed during culmination, suggesting the existence of an inducing signal originating from the apical, prestalk, cells (Richardson et al., 1994). Production of this signal may require the action of the tagB/tagC gene products (Shaulsky et al., 1995).

Conditions have been established in which spores and stalk cells can be formed in cell monolayers in the absence of multicellularity (Town et al., 1976; Berks and Kay, 1988). Using this approach, it is possible to analyse the mode of action of substances that influence cell differentiation. cAMP is required for the expression of prestalk genes but inhibits terminal stalk formation (Berks and Kay, 1988). DIF-1 was detected and characterized by its ability to induce stalk formation (Town et al., 1976; Kay and Jermyn, 1983). In Ax2 and its derivatives, stalk cell formation is most efficient if the cells are first starved in the presence of cAMP to induce prestalk cells, then washed and incubated with DIF-1 (Berks and Kay, 1988). Stalk cell formation is more efficient in V12M2 and its derivatives, which also have much reduced sensitivity to inhibition by cAMP (Town et al., 1976; Sobolewski et al., 1988; Berks and Kay, 1988). Wild-type cells can differentiate into prespore cells if starved under submerged conditions in the presence of a high concentration of cAMP but they will not become mature spores (Kay and Jermyn, 1983; Oohata, 1995). Three complementation groups of mutants

have been identified, *rde*A, *rde*C and regA, that are able to form spores under these conditions (Abe and Yanagisawa, 1983; Kay, 1989; Kessin, 1977; Shaulsky et al., 1996). All three also display rapid development. The rapid development and sporogeny seem to depend in each case on increased levels of cyclic AMP and /or of cAMP-dependent protein kinase (PKA) activity. Thus there is evidence that rdeA as well as regA$^-$ cells lack an intracellular cAMP-specific phosphodiesterase activity (Shaulsky et al., 1996; Chang et al., 1996) while *rdeC* mutants have constitutively active PKA due to a defect in the PKA regulatory subunit (Simon et al., 1992). The K-P strain, a transgenic line that contains multiple copies of the PKA catalytic subunit gene, is also sporogenous and mimics the *rde*C phenotype (Anjard et al., 1992).

Using K-P cells starved at low density as sensors for spore-inducing factors produced by high-density cultures, two peptides, SDF-1 and SDF-2, have been identified that promote terminal spore differentiation in vitro (Anjard et al., 1997, 1998). SDF-1, a small, PKA-phosphorylated peptide was detected in the supernatant of the K-P strain itself (Anjard et al., 1997), while SDF-2 was isolated from regA$^-$ supernatants. The SDF-2 pathway appears to be largely independent of the SDF-1 pathway and has been analysed in more detail (Anjard et al., 1998). TagC, a prestalk-specific composite serine protease/ATP-driven transporter (Shaulsky et al., 1995) appears to be involved in the maturation and secretion of SDF-2 (Anjard et al., 1998). Exposure of K-P cells incubated at low cell density to SDF-2 induces rapid release of SDF-2 (Anjard et al., 1998) and this factor seems to act on prespore cells via the *dhkA*-encoded receptor/histidine kinase (Wang et al., 1996). Evidence has been presented that both SDF-1 and SDF-2 require PKA for the induction of spore differentiation but that SDF-2 acts by a rapid and apparently direct induction of PKA while SDF-1 evokes a slow and probably indirect response (Anjard et al., 1997, 1998).

Previous work on the production of SDF factors was performed using mutant strains in submerged culture. Here we show that the two SDFs are produced and secreted at distinct times in normal development and can induce cells dissociated from wild-type culminates to form spores under submerged conditions. We also show that the SDFs can influence stalk cell formation under standard submerged conditions (Kay, 1989). Each SDF appears to be released in a single burst that may define an irreversible step in cell differentiation.

MATERIALS AND METHODS

Cells and culture conditions

AX2 is a wild-type axenic strain derived from NC4 (Watts and Ashworth, 1970). The K-P strain is a transformant of Ax2 that carries multiple copies of the *Pka*C gene, specifying the catalytic subunit of cAMP-dependent protein kinase, regulated by its endogenous upstream region (Anjard et al., 1992). *StkA*$^-$/K is the K-P strain in which the *stk* gene has been disrupted by homologous recombination (Chang et al., 1996). Cells were grown in shaking culture in axenic medium with antibiotics and were collected during the exponential phase of growth. V12M2 and HM44 are non-axenic strains derived from an independent natural isolate (strain V12). They were grown in 20 mM phosphate buffer (pH 6.0) suspension culture with *Klebsiella aerogenes* as a food source (Kopachik et al., 1983).

Purification of SDF-1 and SDF-2

SDF-1 was purified from the supernatant of 10^{10} cells that overexpress the catalytic subunit of PKA from the prestalk-specific EcmA promoter (EcmA-PKA, Hopper et al., 1993) starved for 24 hours in 1 litre cAMP buffer (0.2 mM cAMP). cAMP buffer was 10 mM MES pH 6.5, 10 mM NaCl, 10 mM KCl, 1 mM $CaCl_2$ and 1 mM $MgSO_4$ and contained the amounts of cAMP indicated in brackets. Cells and debris were removed by centrifugation (2,000 ***g***, 20 minutes) and filtration (0.45 μm nitrocellulose filter. The cleared supernatant was passed over a cation exchange C5 Sartobond filter (Sartorius), the filter was washed with with 10 mM phosphate buffer (PB) pH 6.0, and 10 mM PB pH 6.0 + 100 mM NaCl. SDF-1 was then eluted stepwise with 5 ml 10 mM PB/1 M NaCl. The sample was filtered through a Filtron Macrosep 10k, diluted 10-fold in PB buffer (pH 6.0) to reduce the salt concentration and then loaded on a resource S column (Pharmacia). After washing, SDF-1 was eluted with a 0 to1 M NaCl gradient in 10 mM PB. The activity elutes at approximately 450 mM NaCl. The sample was diluted 3- to 5-fold in carbonate buffer (20 mM carbonate final concentration, pH 10.2) before loading on a miniQ column (SMART system, Pharmacia). After washing, SDF-1 was eluted with a 0 to 1 M NaCl gradient in 20 mM carbonate buffer (pH 10.2). The activity eluted at 200 to 250 mM NaCl in a final volume of 150 ml. Aliquots of 50 ml were loaded onto a superose 12 gel filtration column equilibrated with 5 mM PB (SMART system). SDF-1 activity eluted as a single peak around 1.1×10^3 M_r based on column calibration (Fig. 1A). More than 50% of the initial activity was recovered from the gel filtration. The activity did not bind to a reverse-phase HPLC C18 column. No SDF-2 activity was found in the supernatant of EcmA-PKA cells nor in the flow through of later purification steps (detection limit in spore differentiation assays was approx. 5×10^{-4} units/10^3 cells). Also, no spore-inducing activity binding to an anion exchange filter (see below; purification of SDF-2) could be detected. Stocks of SDF-1 used for the present study are dilutions of the gel filtration fraction (approximately 1000-fold) and contain 5000 units SDF-1/ml in cAMP buffer (without cAMP) with 100 μg/ml BSA for stabilization. Stocks are stored at –20°C.

SDF-2 was purified from the supernatant of 10^{10} regA$^-$ cells starved for 24 hours in 1 litre of cAMP buffer (0.2 mM cAMP). Cells and debris were removed by centrifugation (2,000 ***g***, 20 minutes) and filtration (0.45 mm nitrocellulose filter). The cleared supernatant was passed over an anion exchange A5 Sartobond filter (Sartorius), washed with 10 mM PB, 10 mM PB + 100 mM NaCl and eluted stepwise with 5 ml 10 mM PB + 1 M NaCl. The sample was filtered through a Filtron Macrosep 10k, diluted 10-fold in PB buffer (pH 6.0) to reduce the salt concentration and then loaded on a miniQ column (SMART system). After washing, SDF-2 was eluted with a 0 to 1 M NaCl gradient in 10 mM PB (pH 6.0). The activity eluted as a single peak around 350 mM NaCl in a final volume of 150-200 ml. Aliquots of 50 ml were loaded on a gel filtration superose 12 equilibrated with 5 mM PB + 150 mM NaCl (SMART system). Part of the SDF-2 activity eluted as a peak around 1.3×10^3 M_r while another fraction eluted at an apparent higher molecular mass together with a high salt fraction. When the high M_r fractions were reconcentrated and run again on superose 12 in a high salt buffer (upto 500mM NaCl), all the activity eluted as a single peak at 1.3×10^3 M_r. The SDF-2 sample was diluted 2- to 5-fold and 0.1% TFA was added before loading on a reverse-phase HPLC C18 column (Vydac). Elution was performed with a 0 to 70% water/acetonitrile gradient with 0.1% TFA. SDF-2 activity eluted as a single peak at 38% to 40% acetonitrile, indicating strong hydrophobicity (Fig. 1B). No SDF-1 activity was found in the supernatant of regA$^-$ cells or in the flow through of later purification steps (detection limit in spore differentiation assays was approx. 5×10^{-4} units/10^3 cells). Also, no spore-inducing activity binding to a cation exchange filter (see above, purification of SDF-1) could be detected. SDF-2 used for the present study was a dilution (approximately 10000-fold) from the HPLC fraction and contained 5000 units SDF-2/ml in cAMP buffer (without cAMP) with 100 μg/ml BSA for stabilization.

Trapping of SDFs

Cation and anion exchange resins (phosphocellulose, Sigma C-2258 and DE52, Whatman N° 4057050) were used to trap SDF-1 and SDF-

2, respectively, to determine factor concentrations in cell supernatants. The resins were pre-equilibrated and resuspended in 1 volume of cAMP buffer (without cAMP). Supernatants of developing cells were incubated with either 10 ml cation or 10 ml anion exchange resin. After 2 minutes of incubation, the resin was pelleted by centrifugation and the supernatant harvested for quantification. Under the conditions used (pH 6.5), the supernatant from medium incubated with cation exchange resin was free of SDF-1 while the one incubated with anion exchange resin was free of SDF-2. Both factors could be quantitatively eluted from the respective ion exchange resins by 1 M NaCl.

The properties of SDF-1 and SDF-2 are summarized in Table 1.

Sporogenous assay

Cells were incubated in submerged culture and sporulation monitored as described previously (Kay, 1987; Anjard et al., 1997). In the sporogenous assay, washed cells were resuspended and plated at the indicated densities in 1.5 cm diameter plastic wells with 0.5 ml of cAMP buffer (5 mM cAMP). Spores were scored microscopically after 20 to 24 hours of incubation at 22ϒC (Anjard et al., 1997). Purified factors were then added to cells and spores counted again 2 hours later. Each assay was repeated at least 3 times and more than 200 cells were scored for every data point. Averaged values are presented ±1 s.e.m. Every sporogenous assay included a positive control for SDF-1 and SDF-2 activity. One unit of SDF-2 activity was defined as the amount necessary to induce K-P cells to form 50% spores. Similarly, one unit of SDF-1 activity was defined as the amount necessary to obtain 35% spores.

To determine concentrations of SDF-1 and SDF-2 in developing cells, Ax2 cells were starved on nitrocellulose filters (5×10^6 or 10^7 cells/filter) as described (Anjard et al., 1992). At different developmental stages filters were placed in an Eppendorf tube with 1 ml buffer without cAMP (see Fig. 4-I). Structures were disaggregated by vigorous shaking for 10 seconds, filters were removed and cells pelleted by 1 minute centrifugation at 4000 revs/minute. Supernatants were harvested for quantification of SDFs. Cells were washed in 1 ml buffer (without cAMP) and plated at $2\times10^4/cm^2$ in 1.5 cm diameter wells with 0.5 ml cAMP buffer (5 mM cAMP). After 10 minutes of incubation, 10 units of SDF-1 and/or SDF-2 were added for priming and aliquots of the supernatants were harvested 5 minutes later to quantify the released factors. As a control, supernatant from non-induced cells was analysed for spontaneous release of SDFs. The level of spores in the disaggregated structures was scored after incubation for 2 additional hours.

Quantification of SDFs was performed by sporogenous assays on K-P cells. The limit of detection was 0.5 units, which still resulted in significant induction of spore formation (Anjard et al., 1998 and data not shown). Assays were performed in three steps: first 1 μl of the sample was tested, if no induction occurred, higher volumes up to 20 or 50 μl were tested. If the 1μl sample resulted in maximal spore formation 10-fold dilutions were assayed to determine the range of SDFs content. In the third step, 1, 2, 5 and 10 μl of the appropriate dilution were tested to obtain a more exact value for the concentration of the factor. Finally, data were standardized to units released by 10^3 cells.

Stalk differentiation assay

Stalk differentiation was performed essentially as described (Kay, 1987; Sobolewski et al., 1988). Cells were grown in axenic media, or in bacterial suspension for V12M2 and HM44, washed and plated at a density of $2\times10^4/cm^2$ in cAMP buffer (0.5 mM cAMP), antibiotics (50 μg/ml Ampicillin, 20 μg/ml Streptomycin) were added. After 20 hours, supernatants were carefully removed and replaced by fresh buffer (without cAMP). The effect of the different factors was assayed by adding 0.2 mM cAMP, 100 nM DIF-1, 100 units SDF-1 and 100 units SDF-2 as indicated. Stalk cell formation was scored by phase microscopy at the times indicated. A cell was considered as stalk when vacuoles corresponded to more than 50% of the cell surface (Chang et al., 1996). Experiments were performed three times and at least 200 cells were scored. Error bars represent ±1 s.e.m.

RESULTS

SDF-1 priming results in a rapid release of SDF-1 in K-P cells

SDF-1 was originally detected as a factor released into the medium by high-density K-P cells that induced the same cells

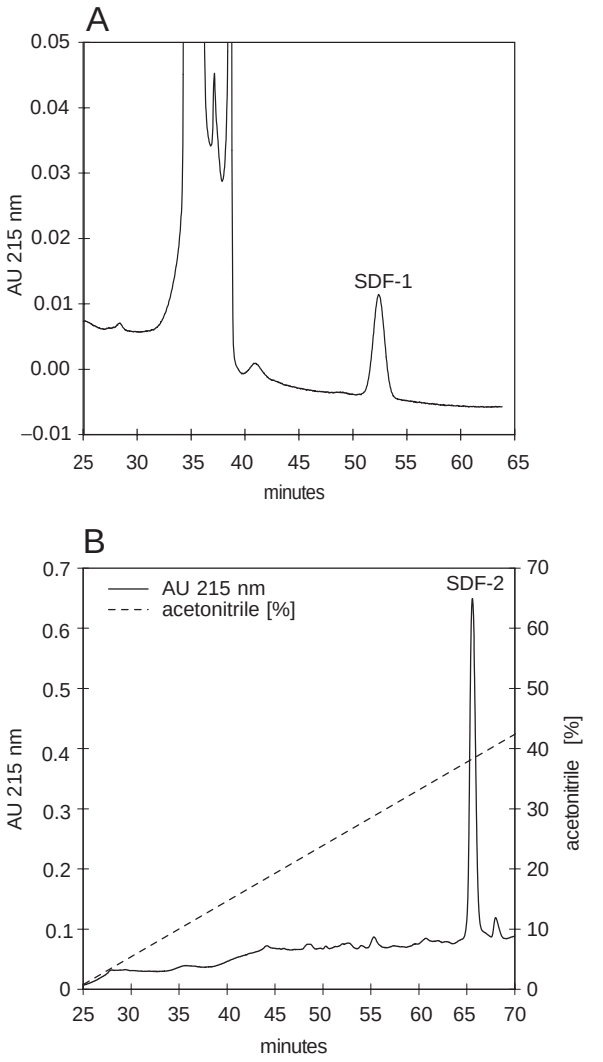


Fig. 1. Purification of SDF-1 and SDF-2. (A) After cation exchange filtration, resource S column chromatography and miniQ column chromatography, the SDF-1 preparation was loaded on a superose 12 gel filtration column equilibrated with 5 mM PB. SDF-1 activity eluted as a single peak at 1.1×10^3 M_r based on column calibration with low molecular weight standards. The figure shows the elution profile at a flow rate of 50 μl/minute. The three combined peak fractions (75 μl each) had more than 10^6 units per μl while surrounding negative fractions had less than 500 U/μl (detection limit in the assay used). No other peak of activity was found in the eluate. (B) After anion exchange filtration, miniQ column chromatography and gel filtration, the SDF-2 preparation was loaded on a C18 reverse-phase HPLC column and eluted with a 0 to 70% acetonitrile gradient in the presence of 0.1% TFA. The figure shows the elution profile at a flow rate of 100 μl/minute. The line representing the gradient was adjusted to compensate for the void volume. The two combined peak fractions (100 μl each) had more than 10^6 units per μl while surrounding negative fractions had less than 500 U/μl (detection limit in the assay used). No other peak of activity was found in the eluate.

Table 1. Summary of characteristics for SDF-1 and SDF-2 as deduced from spore induction experiments and the purification procedure

SDF-1	SDF-2
Induces spore formation in K-P cells in 90 minutes after a delay of 45 minutes	Induces spore formation in K-P cells in 30 minutes after a delay of 5 minutes
M_r of about 1.1×10^3, pI of 8.4	M_r of 1.3×10^3
Activity sensitive to alkaline phosphatase	Activity insensitive to alkaline phosphatase
Binding to cation but not to anion exchange resin at pH 6.0, binding to anion exchange resin at pH 10.2	No binding to cation exchange resin (down to pH 2.5), binding to anion exchange resin at pH 6.0
No or weak binding to C18 reverse-phase HPLC column	Strong binding to C18 reverse-phase HPLC column
Sensitive to trypsin, insensitive to endoproteinase Glu-C and pepsin	Insensitive to trypsin, sensitive to endoproteinase Glu-C and pepsin

incubated at low density to differentiate into mature spores in the presence of cAMP (Anjard et al., 1997). Similarly, SDF-2 was a spore-inducing activity produced by regA$^-$ cells. We have previously shown that K-P cells do not release SDF-2 spontaneously but that exposure to SDF-2 results in the release of the accumulated factor within 2 minutes. This SDF-2-induced release of SDF-2 is referred to as priming. Before examining the accumulation of these factors during normal development of Ax2 cells, we determined whether release of SDF-1 could also be primed. When K-P were starved at low density, no SDF-1 could be detected. Addition of 10 units of SDF-1 to the low-density cells induced rapid release (Fig. 2). It should be noted that SDF-1 and SDF2 each only induce their own release (Anjard et al., 1998 and data not shown).

SDF-1 production correlates with prestalk-specific characteristics

It was shown previously that tagC$^-$ cells do not produce SDF-2 (Anjard et al., 1998). Since tagC is only expressed in prestalk cells (Shaulsky et al., 1995), this suggested that SDF-2 is a prestalk-cell-specific product. We were interested to know whether SDF-1 was also prestalk-cell-specific. We therefore examined the effect of preincubation with DIF-1 on the accumulation of SDF-1 under submerged conditions (Fig. 3). Ax2 cells did not release SDF-1 spontaneously or upon priming when starved for 20 hours at high density in the presence of 200 μM cAMP (Fig. 3 column 1 and data not shown). Nor was SDF-1 released when the cells were incubated for further 20 hours with various concentrations of DIF-1 (column 2). However, cells exposed to 100 pM or more DIF-1 did release SDF-1 when primed (columns 5-8). Thus, addition of DIF-1, a known inducer of prestalk-specific gene expression, resulted in the production of SDF-1 which could be released by priming.

The conclusion from this experiment is supported by the finding that cells expressing PKA-C under the control of the prestalk-specific EcmA promoter (Hopper et al., 1993) release

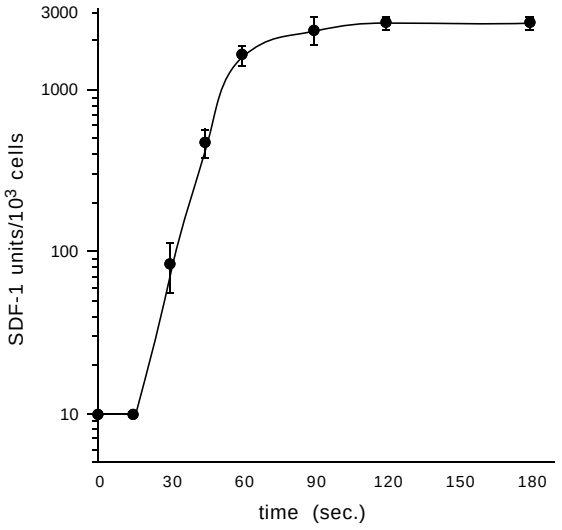


Fig. 2. SDF-1 release upon priming in K-P cells. K-P cells were starved at low density for 24 hours in cAMP buffer (5 mM cAMP). 10 units of SDF-1 were added and the plates were briefly shaken. Aliquots of supernatants were harvested at different times for quantification of released SDF-1 by serial dilution. The error bar corresponds to the standard deviation of three independent time courses.

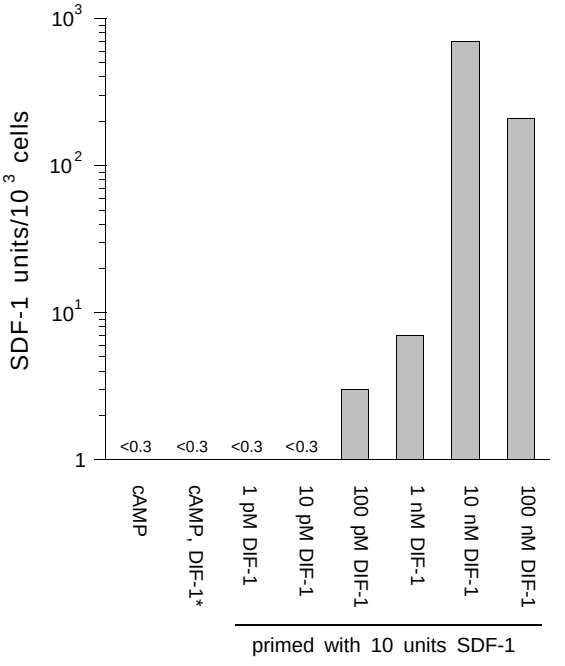


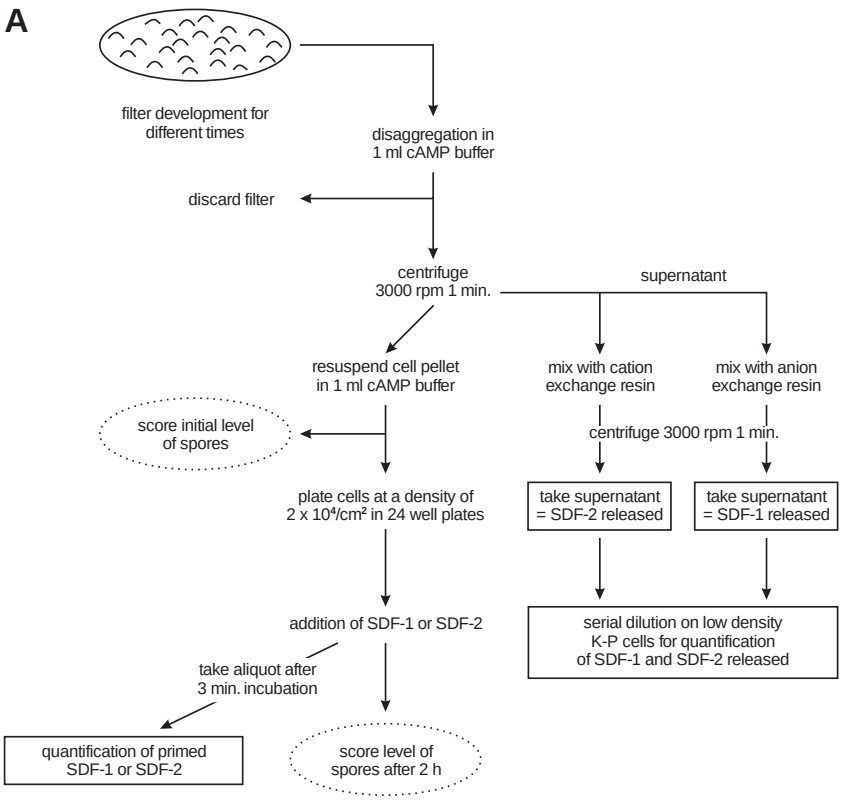
Fig. 3. Release of SDF-1 by AX2. Ax2 cells were starved at 2×10^4/cm^2 under various conditions and supernatants assayed for SDF-1 content by serial dilution on K-P cells. Column 1, Ax2 cells starved for 20 hours with 0.5 mM cAMP; column 2, Ax2 cells starved for 20 hours with 0.5 mM cAMP as before, then the medium was replaced with buffer containing 1 pM to 100 nM DIF-1 as indicated; SDF-1 in the supernatant was determined after a further 20 hours of incubation. Columns 3-8, 10 U SDF-1 added to cells that had been incubated (for 20 hours) with DIF-1 concentrations as indicated, supernatants were prepared 5 minutes later for SDF-1 quantification. *This value was the same for all DIF-1 concentrations examined (1 pM to 100 nM) and is therefore represented by a single column.

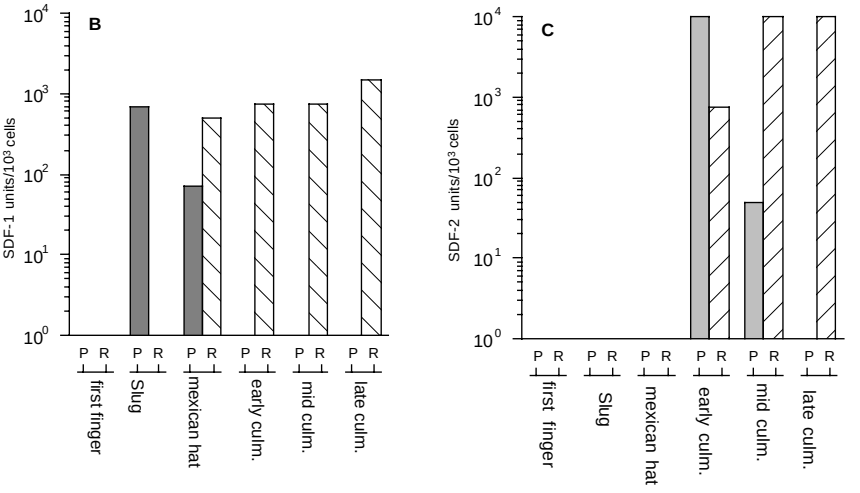
10-fold more SDF-1 than K-P cells (data not shown).

Time course of SDF-1 and SDF-2 production and release

To determine when the SDFs are produced and released during development, Ax2 aggregates were harvested at successive times from filters, disaggregated in cAMP buffer and the concentrations of SDF-1 and SDF-2 in the supernatants was determined separately (Fig. 4; SDFs released). In addition the dissociated cells were resuspended in fresh cAMP buffer, plated and primed by SDF-1 or SDF-2. Supernatants were prepared after 2 to 3 minutes and again assayed for SDF-1 and SDF-2 (SDFs primed). Finally the extent of spore formation by the cells was determined both before and 2 hours after priming (see legend to Fig. 4 for details).

No SDFs were released prior to the slug stage. SDF-1 was first detected after priming the disaggregated slug cells, indicating that the factor had been made but had not yet been secreted (Fig. 4B). SDF-1 was released spontaneously at about the Mexican-hat stage, corresponding to the onset of terminal differentiation. Since the Mexican-hat stage is brief, it was difficult to obtain conditions where the aggregates were synchronised at this stage. Therefore to confirm that SDF-1 is released at the transition to culmination, individual Mexican-hats were harvested and assayed. In the supernatants of three individual Mexican-hats, SDF-1 values ranged from 5×10^2 to 2×10^3 units per 10^3 cells (data not shown) and was thus consistent with the measurement from mass culture on





D

		First finger (14 h)	Slug (16h)	Mexican hat (18 h)	Early culminant (19 h)	mid-culminant (21 h)	late culminant (22-23 h)
SDF-1	released	<0.002	< 0.002	500	500-1000	500-1000	1000-2000
	primed	<0.1	700	70	<0.1	<0.1	<0.1
	Total	<0.1	700	570	500-1000	500-1000	1000-2000
SDF-2	released	<0.002	<0.002	<0.002	500-1000	10000	10000
	primed	<0.1	<0.1	<0.1	6000-14000	30-70	<0.1
	Total	<0.1	<0.1	<0.1	6500-15000	10000	10000
% spore	initial	0	0	0.7	13.4	26.5	76.7
	No addition	0	0.9	2.4	23.1	27.6	≥ 80
	+ SDF-1	0	2	10.5	53.4	60.1	≥ 80
	+ SDF-2	0	1.8	5	52.5	57.8	≥ 80

Fig. 4. Time course of SDFs production and effect in developing Ax2. (A) Ax2 cells were developed on black nitrocellulose filters and harvested at the times indicated. (D) Filters were placed in an Eppendorf tube with cAMP buffer and cells were shaken off, pelleted and supernatants were kept for SDF quantification. In separate aliquots of the supernatant, SDF-1 was trapped by cation exchange resin and SDF-2 by anion exchange resin. The cells were scored for spore levels after washing, then plated at high density and allowed to recover for 10 minutes before addition of 10 units SDF-1 or SDF-2 for priming. Aliquots of supernatants were harvested 3 minutes later to quantify released SDFs. Spore levels were scored again after 2 hours incubation; later counting showed no further increase. (B) Quantification of SDF-1, P corresponds to factor released upon priming; R to factor released in the supernatant of disaggregated structures. (C) Quantification of SDF-2. (D) Absolute values for SDF-1 and SDF-2 production and release during development and percentage of spore formation.

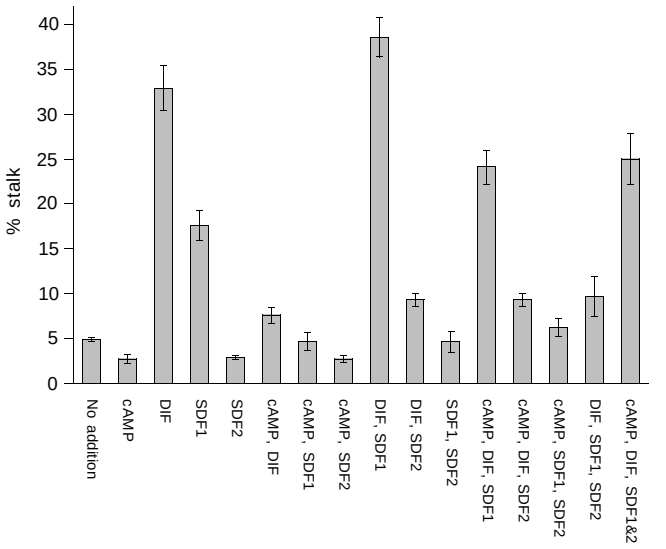


Fig. 5. Combined effects of cAMP, DIF-1 and SDFs on Ax2 stalk formation. AX2 cells were starved for 20 hours at a density of $2\times10^4/cm^2$ in 0.5 mM cAMP buffer and then, after removal of the buffer, incubated with various combinations of DIF-1 (100 nM), cAMP (0.2 mM), SDF-1 (100 U) and SDF-2 (100 U) as indicated for an additional 20-24 hours. Stalk cells were scored under the microscope. Columns represent the mean values of three experiments.

filters. Supernatants from disaggregated structures at later developmental stages had constant amounts of SDF-1 and no further SDF-1 could be released by priming. It appears therefore that SDF-1 is made at the slug stage and secreted in a single burst at the Mexican-hat stage.

SDF-2 could not be detected before early culmination (Fig. 4C). In early culminants, around 10% of total SDF-2 had already been secreted while 90% could be released upon priming. At mid-culminant stage, most SDF-2 had been released and no further accumulation was observed. Supernatants from old fruiting bodies (48 hours after the onset of starvation) showed similar levels of both SDFs as supernatants from late culminants, suggesting no further production or degradation (data not shown).

Some variation in the measured amounts of factors was found in different time-course experiments, especially during the culmination process. This was due to inhomogeneity of development. The time course shown in Fig. 4B,C displays the typical pattern of SDF accumulation and release when development was well synchronized.

Spore formation by dissociated cells in response to SDFs

Cells from disaggregated first finger or earlier stages could not be induced to form spores by either factor (Fig. 4D). Significant induction by either SDFs was first obtained at the Mexican-hat to mid-culminant stage, and increased at later times. This suggests the existence of regulatory pathways controlling responsiveness to the factors.

Effects of SDFs on stalk cell formation in vitro

Since we have shown that the SDFs trigger their own release from prestalk cells (Fig. 2 and Anjard et. al., 1998), it was of

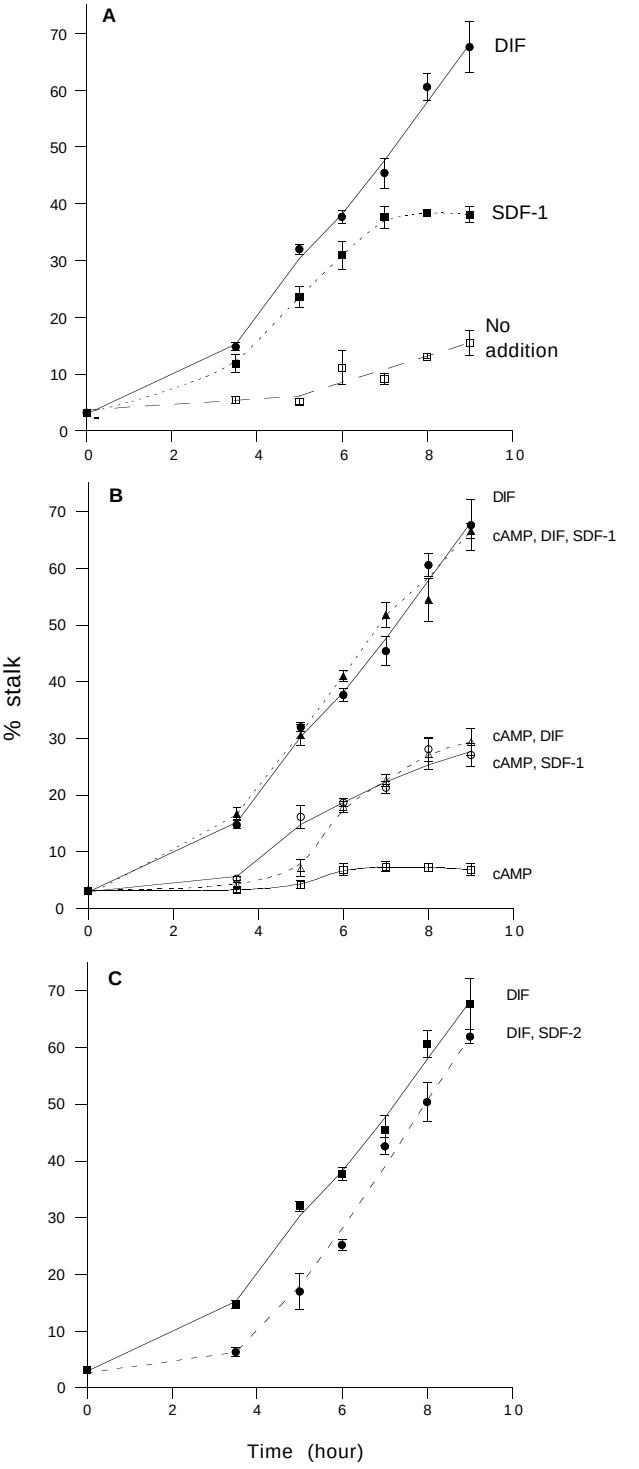


Fig. 6. Time course of stalk formation in *stkA*$^-$/K cells. *StkA*$^-$/K cells were starved for 20 hours at a density of $2\times10^4/ cm^2$ in cAMP buffer (0.5 mM cAMP) and then, after removal of the buffer, incubated with various combinations of DIF-1, cAMP, SDF-1 and SDF-2 (amounts as in Fig. 5) as indicated. The proportions of stalk cells were scored during the following 10 hours. (A) Stalk formation after addition of 100 nM DIF-1, 100 U SDF-1 and without additions. (B) Stalk formation after addition of 100 nM DIF-1, 100 nM DIF-1 and 0.2 mM cAMP and 100 U SDF-1, 0.2 mM cAMP and 100 nM DIF-1, 0.2 mM cAMP and 100 U SDF-1. (C) Stalk formation after addition of 100 nM DIF-1, 100 nM DIF-1 and 100 U SDF-2.

interest to test whether they also influence stalk cell differentiation. Ax2 cells were starved for 20 hours at a density of 2×10^4/ cm^2 in cAMP buffer and then incubated with various combinations of DIF-1 (100 nM), cAMP (0.2 mM), SDF-1 (100 U) and SDF-2 (100 U) as indicated for an additional 20-24 hours (Sobolewski et al., 1988; Berks and Kay, 1988). Stalk cell formation by the Ax2 cells in response to DIF-1 was relatively inefficient; after 20 hours of incubation with DIF-1, there were only 30% stalk cells, and most of them were only partially vacuolated. SDF-1 alone appeared to induce significant stalk cell formation whereas SDF-2 alone had no effect (Fig. 5). Addition of both DIF-1 and SDF-1 resulted in slightly higher levels of stalk cells than incubation with DIF-1 alone. cAMP blocked DIF-1-dependent stalk formation as previously described (Berks and Kay, 1988), and it also blocked SDF-1-dependent stalk cell formation. However, the combination of DIF-1 and SDF-1 was almost resistant to inhibition by cAMP. SDF-2 appeared to reduce stalk cell formation in response to either DIF-1 or SDF-1 but, at the same time, the stalk cells that were formed displayed more complete and homogenous vacuolation (data not shown). Thus SDF-1 promotes stalk cell differentiation and renders the response to DIF-1 resistant to cAMP inhibition, whereas SDF-2 appears to inhibit stalk cell differentiation.

Stalk cell formation by K-P cells was affected by the SDFs in a manner similar to Ax2, but as they also formed spores they did not seem suitable for further analysis. Instead, detailed assays were performed with a *stkA*$^-$ derivative of the K-P strain (*stkA*$^-$/K; Chang et al., 1996) unable to form spores due to a defect in spore maturation. In these experiments, stalk cell formation was monitored over a 9 hour period after washing the cAMP-pretreated *stkA*$^-$/K cells and exposing them to different combinations of regulatory substances. In the presence of DIF-1, the number of stalk cells increased rapidly after 3 hours to reach 60-70% after 9 hours (Fig. 6A), overnight incubation did not result in any further increase (data not shown). Again SDF-1 on its own induced stalk cells though less efficiently than DIF-1, cAMP reduced this response, while the combination of SDF-1 and DIF-1 conferred resistance to cAMP inhibition. Thus the effects of SDF-1 on *stkA*$^-$/K cells closely parallelled those on Ax2 cells. However, in contrast, SDF-2 only reduced induction by DIF-1 slightly in the case of *stkA*$^-$/K cells (Fig. 6C) and what effect there was seemed mainly due to a delay in induction.

Observations with additional strains threw some light on the mechanisms underlying the observed effects of the SDFs. V12M2 cells derive from a different wild-type isolate than AX2. They form stalk cells efficiently in submerged culture at high density without requiring exogenous DIF-1 and are only DIF-1-dependent at low density; in addition, they are almost insensitive to the inhibitory effect of cAMP on stalk cell formation (Berks and Kay, 1988). HM44, is a derivative of V12M2 that produces reduced levels of DIF-1 and is therefore dependent even at high density upon added DIF-1 for forming stalk cells in vitro (Kopachik et al., 1983). Two points of interest emerge from a comparison of the effects of various combinations of factors on V12M2 and HM44 (Fig. 7): while SDF-1 alone could strongly induce stalk formation in V12M2 as it did in the axenic cell lines, it did not do so in HM44 (Fig. 7A,B), suggesting that the response to SDF-1 depends upon a cooperative effect between SDF1 and endogenous DIF-1 (see below). In addition both strains appeared to be insensitive to the inhibitory effects of SDF-2 as well as to cAMP, indicating a possible relation between the inhibitory pathways activated by SDF-2 and by extracellular cAMP.

The *dhkA*$^-$ strain could be induced by DIF-1 or SDF-1 (not shown) much like Ax2 (Fig. 7C). However, as expected, SDF-

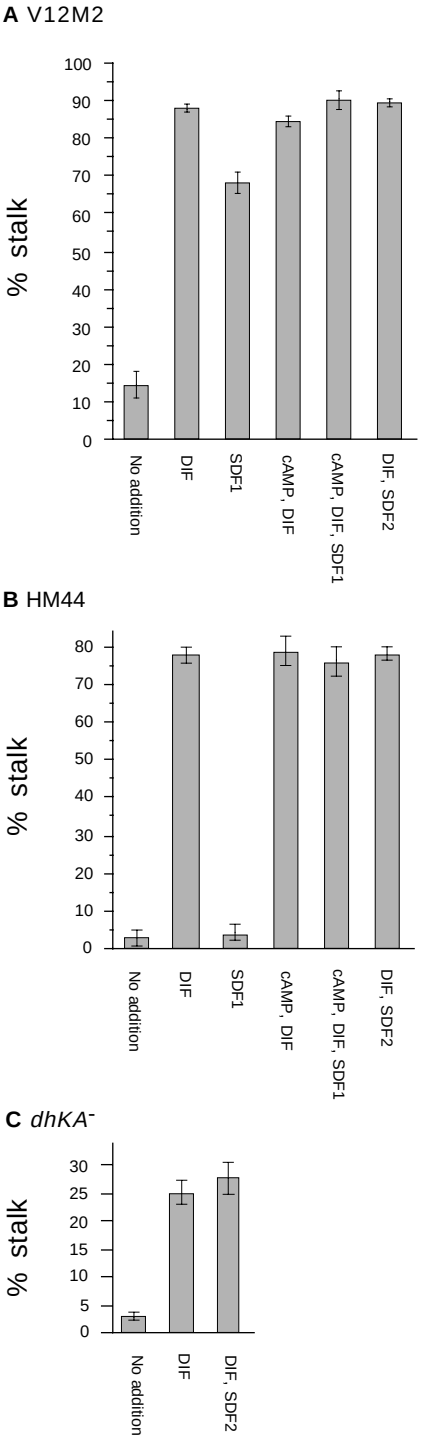


Fig. 7. Effects of SDFs on stalk differentiation of V12M2 and HM44 strains. V12M2 (A), HM44 (B) and *dhkA*$^-$ (C) cells were starved for 20 hours at a density of 2×10^4/cm^2 in cAMP buffer (0.5 mM cAMP) and then incubated with various combinations of DIF-1, cAMP, SDF-1 and SDF-2 (amounts as in Fig. 5) as indicated. The proportions of stalk cells were scored after 10 h. Only relevant combinations of factors are presented.

2 did not reduce the effect of DIF-1 since the dhkA gene product had been suggested to be the receptor for the SDF-2 response (Anjard et al., 1998).

Synergy between SDF1 and DIF-1

To test for the cooperation between SDF1 and DIF-1 inferred above from comparison of the effect of SDF-1 on HM44 and V12M2 cells, we incubated Ax2 and HM44 cells with various concentration of DIF-1 in the presence or absence of 100 units of SDF-1 (Fig. 8A). As shown earlier, the presence of SDF-1 induced some stalk formation in the absence of effective concentrations of DIF-1. More importantly, it shifted the dose-response curve such that the concentration of DIF-1 required for half-maximal stalk formation (IC_{50}) was reduced from about 15 nM to 200 pM DIF-1. Thus SDF-1 synergises with DIF-1.

The experiment was repeated with HM44 cells (Fig. 8B). These required about 10-fold less DIF-1 than Ax2 for half-maximal stalk cell induction and stalk cell formation increased over a much wider range of DIF-1 concentrations. Very similar synergy between SDF-1 and DIF-1 was observed (Fig. 8B) suggesting that their failure to respond to SDF-1 when added on its own may not reflect a basic difference in the responsiveness of the cells but only their inability to produce (sufficient) endogenous DIF-1. Synergy was also observed with V12M2 and *stkA*$^{-}$/K cells (data not shown).

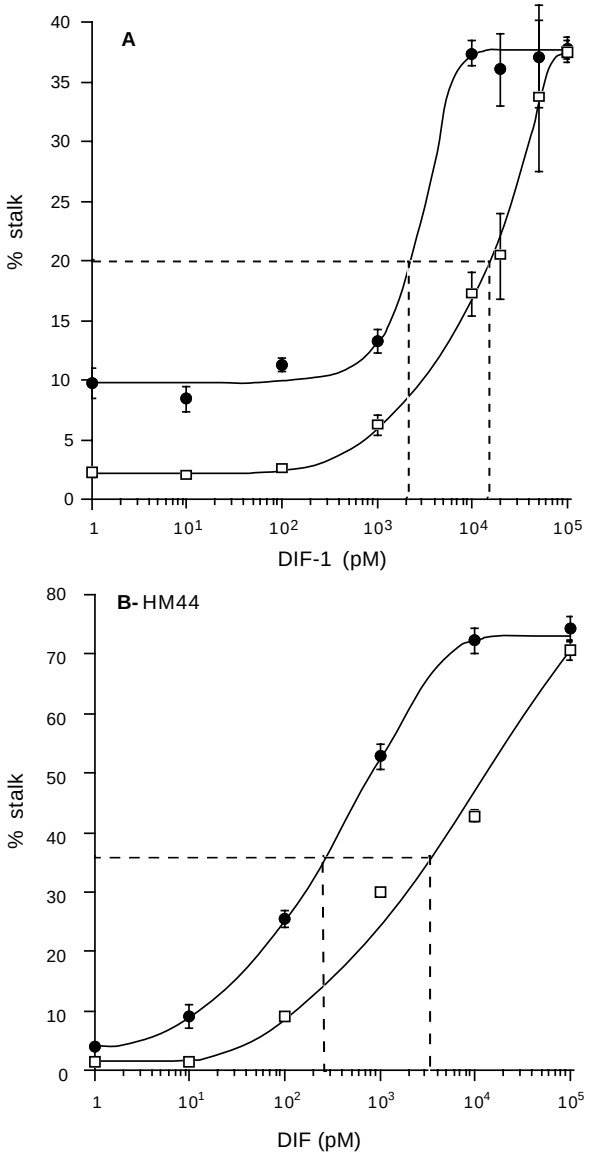


Fig. 8. Synergy of SDF-1 with DIF-1 in stalk cell induction. AX2 (A) and HM44 (B) cells were starved for 20 hours at a density of $2\times10^4/cm^2$ in cAMP buffer (0.5 mM cAMP) and then incubated with various concentration of DIF-1 with (black symbols) or without (white symbols) addition of units of SDF-1. The proportion of stalk cell was scored after 10 hours for HM44 and after 20-24 hours for AX2. Dotted lines indicate half maximal stalk cell formation (IC_{50})

DISCUSSION

As in many other eukaryotes, *Dictyostelium* differentiation is regulated by extracellular signals and probably follows a program with check-points and regulators for the different steps. We have shown that, although SDF-1 and SDF-2 both promote spore formation in low-density monolayers of sporogenous mutants, they accumulate and are secreted at different times during Ax2 development. SDF-1 accumulates during the slug stage and is released when Mexican hats are formed while SDF-2 accumulates during early culmination and is released during late culmination. The release of SDF-1 and then of SDF-2 apparently mark irreversible steps in the developmental program since each factor appears to be released in a single burst, as it is with priming (Fig. 2) and no further accumulation of either factor is detected. Since SDF-1 appears to be produced by prestalk cells and to be liberated at the onset of culmination, it is tempting to suppose that it is involved in actually triggering culmination. This idea gains some support from the analysis of a culmination-defective (slugger) mutant carrying a disruption in a *c-myb* homologue (Kunde Guo, C. A., Adrian Harwood, Peter Newell and J. G., unpublished data). The mutant does not form stalk cells or spores when developing on its own but does so efficiently when mixed with as few as 10% wild-type cells, indicating that its culmination defect can be rescued by a signal emitted by wild-type cells. Direct measurements indicate that the mutant is highly deficient in SDF-1 and SDF-2 production (Kunde Guo, C. A., Adrian Harwood, Peter Newell and J. G., unpublished data). In wild-type cells expressing a β-galactosidase driven by the late spore-specific spiA, a gradient of spore differentiation was observed during the last phase of culmination (Richardson et al., 1994). The first induction occurred at the mid-culmination stage on the apex of the sorocarp and then progressed down during the terminal phase. Thus, the timing of SDF-2 release correlates well with spiA induction and may trigger it. In addition to the difference in the timing of their accumulation and release, SDF-1 and SDF-2 also have distinct effects on stalk cell differentiation in vitro. SDF-1 promotes stalk cell formation whereas SDF-2 inhibits it while improving vacuolisation at the same time. Although it has been suggested that both factors act by stimulating PKA activity (Anjard et al., 1997, 1998), this finding makes it likely that they also function in other intracellular signalling pathways.

SDF-1 seems to have three effects on stalk cell formation: first, it largely abolishes the inhibitory action of cAMP first demonstrated by Berks and Kay (1988), second, it synergizes with DIF-1, i.e. it dramatically reduces the threshold

requirement for DIF-1 for stalk cell formation and, third, it can induce stalk cell formation on its own. This last effect could be a reflection of its synergy with DIF-1 since HM44, a strain producing very low levels of DIF, was the only strain tested that did not make stalk cells in response to SDF-1 alone. However, if that were so the dose-response curve for DIF-1 synergy with SDF-1 (Fig. 8A) would imply that Ax2 cells produce at least 300 pM DIF-1 whereas V12M2 cells, known to generate relatively high levels of DIF-1, only accumulate about 30 pM DIF-1 (Kay, 1989). It is therefore possible that SDF-1 has a bona fide stalk-inducing effect on its own. In that case, it would remain unclear why SDF-1 cannot induce stalk cell formation in HM44. It is worth noting that the V12-derived strains that we have examined (V12M2 and HM44) displayed synergy between SDF1 with DIF-1 but were virtually insensitive to cAMP inhibition of stalk cell formation implying that these SDF-1 effects involve divergent intracellular signalling pathways.

Recently a stalk cell differentiation factor (STIF) was isolated from dissociated slugs incubated in buffer (Yamada et al., 1997). STIF is a small hydrophilic factor that has not been shown to be a peptide. It promotes stalk formation in conjunction with DIF-1 and induces EcmB but not EcmA expression. Furthermore STIF requires PKA activity to exert its effects. It may therefore be identical to SDF-1 but it has not been possible to test this yet since STIF is not available.

We have found that, whereas SDF-2 inhibits or slows down stalk cell differentiation in the various axenic *dhkA*$^+$ strains tested (and seems to increase the extent of their vacuolization), it has no effect in *dhkA* null cells. DhkA is believed to specify a transmembrane histidine kinase and to act in a signalling cascade ultimately stimulating PKA activity (Wang et al., 1996; Anjard et al., 1998). Since SDF-2 did not inhibit stalk cell formation in *dhkA*$^-$ cells, the dhkA receptor is probably responsible for the effect of SDF-2 on stalk cell formation as has been suggested for SDF-2-dependent priming, and for the induction of spore formation (Anjard et al., 1998). SDF-2 is released close to the end of Ax2 development and may therefore act as a trigger for terminal spore differentiation as well as influencing stalk elongation. The abnormal, brittle stalks formed by *dhkA*$^-$ cells (Wang et al., 1996) may support the latter idea. Whether or not this is the case, the further analysis of the effects of these peptide factors promises to throw new light on the mechanisms controlling cell differentiation and morphogenesis in *Dictyostelium*.

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