Chemoattractant-Regulated Mobilization of a Novel Intracellular Compartment in Human Neutrophils

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A novel mobilizable intracellular compartment was identified in human neutrophils by latent alkaline phosphatase activity. This compartment is mobilized to the plasma membrane much more readily than any identified granule subset and has kinetics of up-regulation in the membrane similar to those reported for a variety of receptor proteins. Triton X-100 permeabilization of both intact human neutrophils and subcellular fractions obtained by density-gradient centrifugation revealed that 70 percent of the alkaline phosphatase is located in an intracellular compartment distinct from primary, secondary, and gelatinase granules and from the plasma membrane. This compartment fully translocates to the plasma membrane after stimulation with nanomolar concentrations of the chemotactic peptide *N*-formylmethionylleucylphenylalanine.

IN PHAGOCYTES THE MEMBRANE OF intracellular granules is increasingly recognized as a major store of several proteins whose activity in the intact cell is dependent on plasma membrane localization (*1–5*). Fusion of these intracellular membranes with the plasma membrane, as occurs during degranulation, is believed to be the basis for the increased surface expression of these proteins after stimulation (*3–6*). This translocation of membrane offers a mechanism whereby the activity of these proteins can be controlled.

In human neutrophils the specific granules have been identified as an intracellular store of complement receptor type 3 (CR3), which is identical to the adhesion-promoting glycoprotein Mac-1 (*7–9*), and the receptor for the chemotactic peptide *N*-formylmethionylleucylphenylalanine (fMLP) (*1, 2*). An unexplained discrepancy exists, however, between the efficacy by which nanomolar concentrations of fMLP induce up-regulation of these proteins on the surface of the neutrophils and the inability of the same stimulus to induce significant degranulation of specific granules and translocation of the granule membrane–bound proteins, for example, cytochrome b, to the plasma membrane (*4, 8–11*). Also, the decay accelerating factor (DAF) and complement receptor type 1 (CR1) display the same kinetics of up-regulation on the surface of intact neutrophils as CR3/Mac-1. Yet, their intracellular storage sites have not been identified and appear to be distinct from the secondary granules (*4, 9, 12, 13*).

As we tried to solve these paradoxes, we speculated that there might be a novel intracellular storage site for these proteins in neutrophils. This store should degranulate in response to activation by nanomolar concentrations of fMLP to explain the up-regulation of the above-mentioned proteins, and the subcellular localization on density gradients should be close to that of the plasma membranes to explain the apparent lack of translocation observed on the density gradients after up-regulation. During studies on this problem, we assayed alkaline phosphatase, which is commonly used as a plasma membrane marker in human neutrophils (*2, 3, 7, 8, 10, 11, 14, 15*). We observed that alkaline phosphatase activity is rapidly up-regulated in intact cells in response to nanomolar concentrations of fMLP and, to some extent, after incubation at 37°C alone (Fig. 1). The up-regulation induced by fMLP was independent of cell concentration in the range 10^6 to 10^8 cells per milliliter. In the absence of fMLP stimulation, permeabilization of cells with Triton X-100 revealed latent enzyme activity, and fMLP-stimulated up-regulation of alkaline phosphatase was accompanied by a corresponding loss of latent enzyme activity. Since the *p*-nitrophenyl phosphate substrate for alkaline phosphatase is membrane-impermeable, this suggests that fMLP stimulates translocation of an intracellular pool of alkaline phosphatase to the cell surface. The concentration of fMLP ($10^{-8}M$) that induced up-regulation of approximately 90% of the latent alkaline phosphatase activity caused only minimal degranulation of previously identified mobilizable granules: 5.4% ($n = 6$, range 4 to 7%) secretion of β-glucuronidase, a marker enzyme for azurophil granules (*3, 15, 16*); 21.3% ($n = 6$, range 15 to 24%) secretion of vitamin B-12–binding protein, a marker protein for specific granules (*3, 14, 17*); and 30.2% ($n = 6$, range 18 to 40%) secretion of gelatinase, a marker protein for gelatinase-containing granules (*14*).

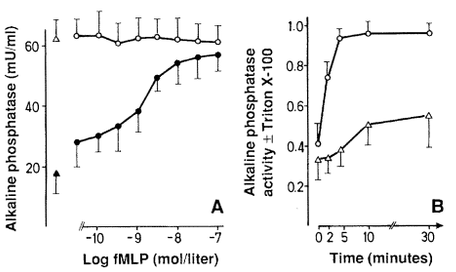

Fig. 1. Neutrophils were isolated by dextran sedimentation at room temperature and density centrifugation on Ficoll-Hypaque in the cold followed by hypotonic lysis of erythrocytes (*3*). (**A**) Purified neutrophils were incubated at 3×10^7 cells per milliliter in KRP buffer (130 m*M* NaCl, 5.0 m*M* KCl, 1.27 m*M* $MgSO_4$, 0.95 m*M* $CaCl_2$, 5.0 m*M* glucose, 10 m*M* sodium phosphate, *p*H 7.4) either at 4°C (▲ and △) or at 37°C (● and ○) with various concentrations of fMLP added from a 1 m*M* stock in ethanol. After 15 minutes, 2 volumes of ice-cold KRP was added, and the cells were sedimented by centrifugation at 200*g* for 8 minutes and resuspended to the original volume of 1 ml in KRP. Alkaline phosphatase was then assayed with *p*-nitrophenyl phosphate as substrate (*23*) in the absence (▲ and ●) or presence (△ and ○) of 0.1% Triton X-100. (**B**) Cells were isolated as above and resuspended in KRP buffer and incubated at 3×10^7 cells per milliliter at 37°C in the presence (○) or absence (△) of $10^{-8}M$ fMLP. At the times indicated, the incubation was stopped and the cells were processed for determination of alkaline phosphatase in the presence or absence of 0.1% Triton X-100 as described above. Results are the mean of three experiments. Bars indicate 1 SD.

To confirm this indication that approximately 70% of the total neutrophil alkaline phosphatase is localized in an intracellular compartment that is mobilized to the plasma membrane by fMLP stimulation, we subjected fMLP-stimulated cells and control cells to subcellular fractionation (Fig. 2, A and B). Tritiated concanavalin A (Con A) was added to the cells before cavitation to identify the plasma membrane (*2*). Measured in the absence of detergent, alkaline phosphatase activity colocalized exactly with the plasma membrane marker ^{3}H-labeled Con A, both in stimulated and unstimulated cells. Addition of 0.1% Triton X-100 to the subcellular fractions from unstimulated cells exposed a large pool of alkaline phosphatase activity. In contrast to the accessible alkaline phosphatase, this pool of latent alkaline phosphatase localized distinctly from the plasma membrane (Fig. 2A). Latent alkaline phosphatase was also distinguished from the primary granules (β-glucuronidase), the secondary granules (B-12–binding protein), and the gelatinase granules. Latent alkaline phosphatase was almost totally absent in fMLP-stimulated cells (Fig. 2B). Similarly, when Triton X-100 was added to the postnuclear supernatants (S_1) that were loaded onto the gradients, alkaline phosphatase ac-

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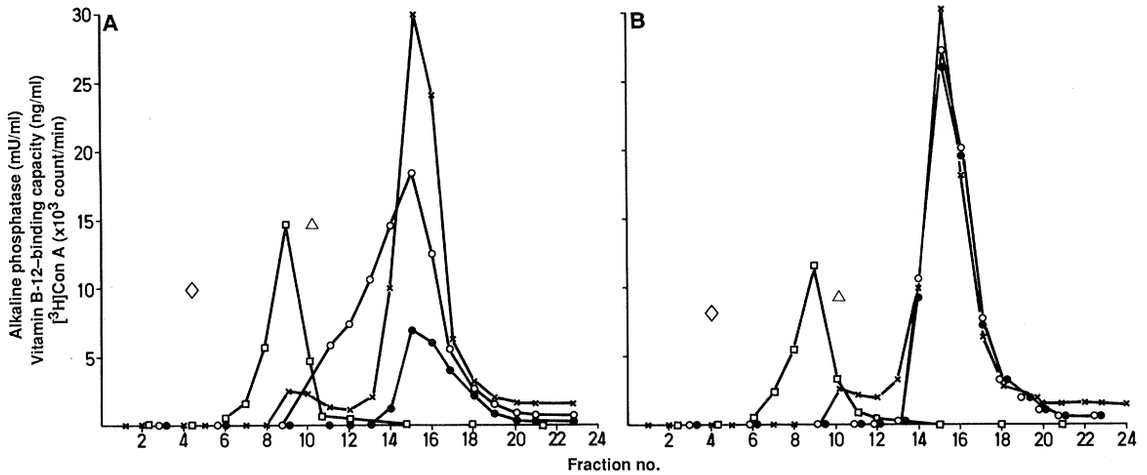

Fig. 2. Neutrophils, 1.2×10^9 isolated as in Fig. 1, were resuspended at 4°C in KRP to a concentration of 5×10^7 cells per milliliter. Fifty microliters of ^{3}H-labeled acetylated Con A (Amersham, 60 Ci/mmol, 50 μCi/ml) was added and the suspension was incubated at 4°C for 10 minutes, and then divided in two. The cells were sedimented by centrifugation and resuspended to a concentration of 5×10^7 cells per milliliter in KRP. One sample was kept at 4°C (**A**), the other was incubated at 37°C with 10^{-8}*M* fMLP (**B**). After 15 minutes, 2 volumes of ice-cold KRP containing 10 m*M* diisopropyl fluorophosphate was added to both samples, and these were further incubated on ice for 10 minutes. The cells were then sedimented by centrifugation and resuspended in 15 ml of relaxation buffer [100 m*M* KCl, 3 m*M* NaCl, 1 m*M* adenosine triphosphate (ATP)Na_2, 3.5 m*M* $MgCl_2$, 10 m*M* 1,4-piperazinediethanesulfonic acid (Pipes), *p*H 7.3] containing 0.5 m*M* phenylmethylsulfonyl fluoride (PMSF) and subjected to nitrogen cavitation (*3*). Ten milliliters of the postnuclear supernatants (S_1) [protein concentration 1.12 mg/ml in (A); 1.08 mg/ml in (B)], were loaded onto two-step 28-ml Percoll density gradients (density 1.05 and 1.12) as described (*3*) except that 0.5 m*M* PMSF was included in the gradients. Fractions of 1.5 ml were collected from the bottom of the gradients and analyzed for alkaline phosphatase in the absence (●) and presence (○) of 0.1% Triton X-100, and for vitamin B-12–binding protein with ^{57}Co-labeled cyanocobalamin (Amersham) (□), β-glucuronidase, and gelatinase. Only peak fractions of β-glucuronidase (◇) and gelatinase (△) are indicated for simplicity. We removed 100 μl from each sample for liquid scintillation counting of ^{3}H-labeled Con A (x). Results of a typical experiment are given. Recovery of material loaded on the gradients was as follows: alkaline phosphatase in the absence of Triton X-100: (A) 108%; (B) 106%; alkaline phosphatase in the presence of Triton X-100: (A) 96%; (B) 101%; vitamin B-12–binding protein: (A) 91%; (B) 88%; ^{3}H-labeled Con A: (A) 91%; (B) 88%; β-glucuronidase: (A) 67%; (B) 71%; gelatinase: (A) 59%; (B) 55%.

tivity was increased 3.65-fold (n = 4, range 2.81 to 4.11) in samples from unstimulated cells but only 1.12-fold (n = 4, range 1.03 to 1.22) in samples from cells stimulated with 10^{-8}*M* fMLP. When added to S_1, fMLP was without effect on alkaline phosphatase activity. These results indicate that alkaline phosphatase is localized on the inside of the membrane of a novel chemoattractant-mobilizable granule subset and translocates to the outside of the plasma membrane after fusion of these granules with the plasma membrane.

Intracellular vesicles that contained alkaline phosphatase were visualized histochemically in 1979 (*18*) and were later separated from the plasma membrane (*19*), but mobilization was not demonstrated, and the significance of these observations was not appreciated. Using the same histochemical technique, others found alkaline phosphatase to be confined to the plasma membrane and its invaginations (*20*). Since then, alkaline phosphatase has been widely accepted as a plasma membrane marker (*2, 3, 7, 8, 10, 11, 14, 15*). This may in part be because some of the procedures involved in isolating neutrophils result in significant up-regulation of alkaline phosphatase activity. We found that neutrophils isolated from buffy coat preparations supplied by a blood bank (Gentofte Hospital) already had 60% of the alkaline phosphatase activity accessible in the absence of detergent. This activity rose to 90% after incubation at 37°C for 15 minutes. Similar cell preparation–induced, temperature-dependent mobilization of CR1 and CR3/Mac-1 has been described (*12, 21*). When neutrophils were isolated from freshly drawn blood as described in Fig. 1 and heated to 37°C, 20 to 35% of the latent alkaline phosphatase activity was translocated (Fig. 1), but, if alkaline phosphatase activity was studied in the leukocyte-rich supernatant of freshly drawn blood after dextran sedimentation of erythrocytes without further purification of the neutrophils, incubation at 37°C for 15 minutes induced no up-regulation (ratio of activity measured in the absence and presence of 0.1% Triton X-100 was 0.25 before incubation and 0.27 after 15 minutes, mean of two experiments). Yet, stimulation with 10^{-8}*M* fMLP induced full up-regulation of alkaline phosphatase (ratio in the absence and presence of Triton: 0.93, mean of two experiments) as measured both in intact cells and in subcellular fractions generated as in Fig. 2.

These findings show that latent alkaline phosphatase identifies a novel mobilizable intracellular compartment in human neutrophils. The kinetics of mobilization of this compartment shows that it cannot be related to endocytotic vesicles (*22*). The localization of this compartment close to, but distinct from, the plasma membrane on Percoll density gradients and the fMLP-induced fusion with the plasma membrane suggest that this compartment serves as an intracellular store of proteins. The up-regulation of this compartment by chemotactic stimuli would thus be important for the function of the neutrophil in adhesion to endothelial cells at inflammatory sites, in chemotaxis, and in the binding and phagocytosis of complement-opsonized microorganisms.

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Hybrid Dysgenesis in *D. melanogaster* Is Not a General Release Mechanism for DNA Transpositions

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Many spontaneous mutations are caused by the insertion or excision of DNA elements. Since most mutations are deleterious, evolution should favor a mechanism for genetically controlling the rate of movement of transposable elements in most, if not all, organisms. In *Drosophila melanogaster* a syndrome of correlated genetic changes, including mutation, chromosome breakage, and sterility, is observed in the hybrid progeny of crosses between different strains. This syndrome, which is termed hybrid dysgenesis, results from the movement of P-DNA elements. What is not clear is whether the movement of other types of transposable elements is under the same coordinated control. In this study the ability of hybrid dysgenesis to increase the rate of excision of 12 DNA elements at 16 mutant alleles and to induce insertion-bearing mutations to change to other mutant states was tested. The data show that hybrid dysgenesis caused by P-element transpositions does not act as a general stimulus for the movement of other Drosophila transposable elements.

TRANSPOSITIONS OF DNA ELEMENTS are a major cause of spontaneous genetic change in many prokaryotic and eukaryotic organisms (*1*). In *Drosophila melanogaster*, where about one-tenth of the genome is mobile, the majority of spontaneous mutant alleles that have been analyzed at the DNA level contain inserts. These inserts have been localized to introns, exons, and regulatory sequences of genes (*2*); when mobilized, these elements are often imprecisely excised (*3*). In addition to gene changes, transposable elements also cause chromosome breakage, nondisjunction, male recombination, and sterility. With this potential for cellular and genetic damage by transpositions, it is not surprising that genetic control of the rate of movement of these nomadic DNA sequences occurs in higher organisms.

An excellent model system for studying this control is the hybrid dysgenesis syndrome in *D. melanogaster*, where a variety of genetic changes (including high frequencies of mutation, chromosome breakage, sterility, and distortion in the ratio of transmission of alleles in gametes) occur in hybrid progeny of controlled crosses between independent population lines (*4, 5*). Frequently, this syndrome results from the insertion and excision of a family of P-DNA elements in germ cells (*6*). Hybrid dysgenesis releases the suppression of P-element movement, which leads to increased production of aberrant genetic traits. Both chromosomal and cytoplasmic components are involved; lines that have complete elements are called *P* lines and lines with no P elements or defective P elements are called *M* or pseudo-*M* lines (*7*).

Although it is clear that mobile DNA elements can disrupt genetic systems, it still remains to be determined whether different transposable elements move under the direction of element-specific enzymes or whether a number of different elements may move under a coordinated control mechanism. For example, a transposase that is coded by one type of element might be used only by these elements for transposition; alternatively, a transposase coded by one element might be used by a number of different elements for transposition. The latter mechanism would have the potential for inducing a larger amount of genetic damage. There is conflicting evidence in the literature on these two possible mechanisms of transposition in *D. melanogaster*. It has been reported that hybrid dysgenesis is a stimulus for the movement of DNA elements other than P, including foldback, copia, mdg, and gypsy sequences (*8*). This suggests that the P-element transposase can be used by other DNA elements, or that P-element–induced genetic damage acts as a general mobilizer of transposons. On the other hand, negative results have been reported for interactions between hybrid dysgenesis and the movement of I, TE, B104, copia, and foldback elements (*9*).

Since transpositions induce a variety of changes that may affect the genetic structure of natural populations, it is clearly important to define any interactions between different mobile elements. This is especially true if different transposons can be activated by similar genetic or environmental events (*10*). In this study we have measured the ability of hybrid dysgenesis in three well-characterized strains of *D. melanogaster* to increase the rate of excision of 12 DNA inserts at 16 mutant alleles. The data from this study do not support the hypothesis that hybrid dysgenesis is a general stimulus for excision of different DNA elements.

We used the natural-population *P* lines *OK1*, *W8D*, and *π2*, and the *M* line Canton-S (*5*). In addition, three *P* lines contained P-element inserts at the white locus ($w^{hd\,81c2}$, $w^{hd\,81b9}$ and $w^{hd\,80k17}$) (*6*). The other lines that carried visible mutations containing DNA inserts were *M* and were obtained from the Mid-America Drosophila Stock Center, except for r^{MR2}, which was obtained from C. Osgood (Old Dominion University, Norfolk, Virginia). The DNA insert at each mutant allele is shown in Table 1. All stocks were tested for *M* or *P* activity by measuring gonadal dysgenesis in hybrid progeny raised at 29°C (*7*). In addition, the *OK1*, *W8D*, *π2*, and Canton-S genomes were probed for P elements by Southern blot analysis; the *OK1*, *W8D*, and *π2* lines contained 20 to 30 DNA sequences that hybridized to the P probe, whereas Canton-S contained eight such sequences that are defective in hybrid dysgenesis activity (Canton-S is therefore a pseudo-*M* line) (*11*).

Mutation events in the absence of hybrid dysgenesis were scored by one of two methods: (i) by mating individual males that contained visible mutations with attached-X (*M*) females (females whose X chromosomes are joined by a single centromere) and screening F1 males for reversions to wild type and for changes to other mutant phenotypes, or (ii) by mating mutation-bearing females to Canton-S (*M*) males, mating individual F1 males to attached-X females, and scoring F2 males for genetic changes. Mutation events in the presence of hybrid dysgenesis were scored by mating

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