Ultraviolet Light and Osmotic Stress: Activation of the JNK Cascade Through Multiple Growth Factor and Cytokine Receptors

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Exposure of mammalian cells to ultraviolet (UV) light or high osmolarity strongly activates the c-Jun amino-terminal protein kinase (JNK) cascade, causing induction of many target genes. Exposure to UV light or osmotic shock induced clustering and internalization of cell surface receptors for epidermal growth factor (EGF), tumor necrosis factor (TNF), and interleukin-1 (IL-1). Activation of the EGF and TNF receptors was also detected biochemically. Whereas activation of each receptor alone resulted in modest activation of JNK, coadministration of EGF, IL-1, and TNF resulted in a strong synergistic response equal to that caused by exposure to osmotic shock or UV light. Inhibition of clustering or receptor down-regulation attenuated both the osmotic shock and UV responses. Physical stresses may perturb the cell surface or alter receptor conformation, thereby subverting signaling pathways normally used by growth factors and cytokines.

Exposure of mammalian cells to UV light results in a gene induction response called the UV response (*1*), which includes the immediate early genes c-*fos* (*2*, *3*) and c-*jun* (*3*) and transcription factors AP-1 and nuclear factor kappa B (NF-κB) (*3*, *4*). Increased transcription of c-*jun* and c-*fos* induced by UV is mediated through phosphorylation of transcription factors c-Jun (*5*), activated transcription factor–2 (ATF2) (*6*), and Elk-1 (*7*) by members of the mitogen-activated protein kinase (MAPK) family, the c-Jun NH_2-terminal kinases (JNKs), also known as stress-activated kinases (*8*).

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JNKs are strongly activated in cells exposed to UV irradiation or osmotic shock (*9*) and are more modestly activated by growth factors and proinflammatory cytokines, including EGF, TNF, and IL-1 (*10*, *11*). EGF leads to JNK activation by a pathway that depends on activation of the small guanosine triphosphate (GTP)–binding proteins Ras (*11*) and Rac (*12*) and the MAPK kinase kinase, MEKK1 (*11*). In turn, MEKK1 phosphorylates and activates the MAPK kinase JNKK1 (MKK4), which activates the JNKs (*13*). The UV-induced signaling cascade is partially dependent on Ras activation (*5*, *14*).

UV irradiation activates Src tyrosine kinases, and tyrosine kinase inhibitors block c-*jun* induction by UV light (*5*). Cell enucleation experiments have ruled out the involvement of a nuclear signal in mediating JNK and NF-κB activation by UV light, suggesting that the initial signal is generated either at the plasma membrane or in the cytoplasm (*15*). Generation of a signal at the plasma membrane would be consistent with the demonstration that UV exposure induces rapid tyrosine phosphorylation of the EGF receptor (EGF-R) (*16*). Although it was proposed to mediate downstream events, the amount of EGF-R tyrosine phosphorylation induced by UV light was low compared with that induced by EGF (*16*). On the other hand, JNK activation by EGF and consequently c-Jun phosphorylation are much weaker than those elicited by UV light (*17*). We therefore considered the possibility that, in addition to activating EGF-R, UV irradiation induces other signaling events that cause potent JNK activation.

At sea level, most of the damaging UV radiation is in the B range (280 to 320 nm). Exposure of various mammalian cell lines to UV-B light also resulted in efficient NF-κB and JNK activation (*18*). Therefore, we used the physiologically more relevant UV-B range, but similar results were also obtained with UV-C light (250 to 280 nm) (*15*, *18*).

Ligand binding to the EGF-R and other growth factor and cytokine receptors, including the TNF receptor (TNF-R) and IL-1 receptor (IL1-R), results in their multimerization, clustering, and internalization (*19*). Receptor multimerization in the absence of ligands was shown to be sufficient for activation of several receptors, including EGF-R and TNF-R (*19*). We thus examined the effect of UV-B irradiation on the aggregation state of EGF-R in HeLa cells by indirect immunofluorescence with receptor-specific antibodies (Fig. 1). Like incu-

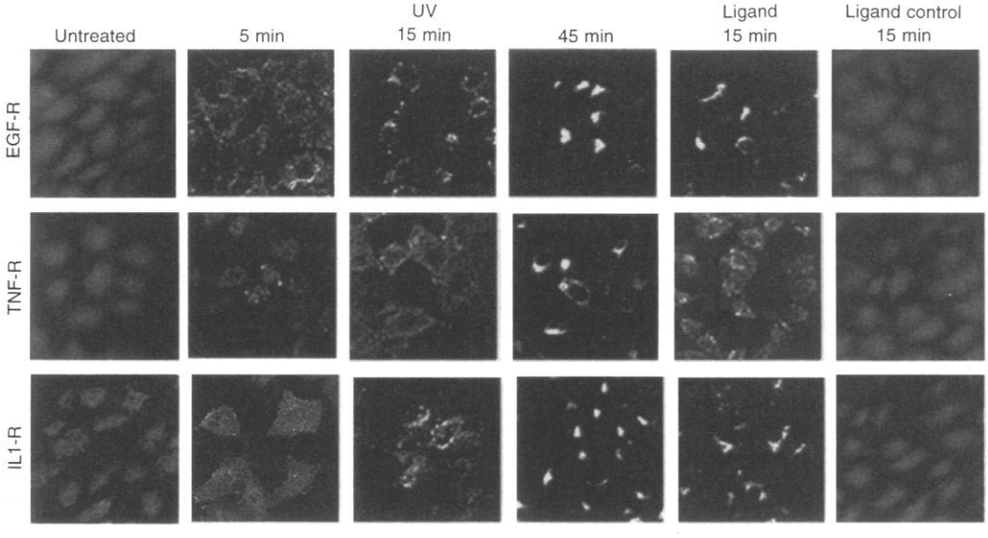

Fig. 1. Effect of UV-B light on aggregation state of the EGF, IL-1, and TNF receptors. HeLa cells cultured on glass cover slips in Dulbecco's minimal essential medium (DMEM) were deprived of serum for 36 hours. Cells were left untreated, incubated with the various ligands (EGF, 100 ng/ml; TNF, 10 ng/ml; IL-1α, 4 ng/ml) (column labeled Ligand) or exposed to UV-B light (600 $J \cdot m^{-2}$, 12 W). At the indicated time points the cells were fixed with 4% paraformaldehyde, treated with 0.1% NP-40 for 5 min, and stained for indirect immunofluorescence with antibodies to each of the three receptors [mAB108 to EGF-R; AB225 to p55 TNF-R, and AB280-NA to IL-1α receptor (the latter two were from R&D Systems, Minneapolis, Minnesota)]. Cells were viewed and photographed with a Bio-Rad MRC1000 confocal microscope with a 40× objective. Specificity of receptor clustering was proven by the inability of the heterologous ligands (for example, TNF for EGF-R or EGF for IL1-R) (column labeled ligand control) to induce receptor clustering (*20*).

bation with EGF itself (*20*), UV-B irradiation resulted in clustering of EGF-R within 5 min. The clusters became larger during the next 10 min. At later time points most of the EGF-R immunoreactivity in either EGF- or UV-B–treated cells was present as very large aggregates, which may reflect internalization of EGF-R to endosomes and lysosomes (*21*). The induction of EGF-R clustering and aggregation was specific, as it was not induced by treatment with either IL-1 or TNF. Chemical cross-linking experiments revealed the formation of EGF-R dimers as early as 1 min after UV-B irradiation (*20*).

Exposure of keratinocytes to UV radiation induces expression of the proinflammatory cytokines IL-1 and TNF (*22*). Both of these cytokines are efficient activators of JNK (*10*), and IL-1 may mediate c-*fos* induction by UV-C light (*16*). Both the IL-1 and TNF promoters contain AP-1 sites and thus may be responsive to JNK activation (*23*). Thus, we also examined the effect of UV-B light on IL1-R and TNF-R (the p55 receptor that is expressed in HeLa cells). In both cases UV-B exposure induced receptor clustering similar to that seen with the cognate ligands, followed by receptor internalization (Fig. 1). These effects were specific because the heterologous ligands did not induce clustering of either IL1-R or TNF-R.

We observed no clear wavelength dependence in induction of signaling responses by either UV-C or UV-B light (*18*). We therefore considered that the UV effect may be produced by a perturbation of membrane structure or a conformational change in membrane proteins resulting from energy absorption (*24*). Another physical stress that causes robust JNK activation and may perturb the membrane is osmotic shock. Osmotic shock, induced by placing HeLa cells in medium containing 600 mM sorbitol, also led to clustering and internalization of EGF-R, IL1-R, and TNF-R (Fig. 2).

One of the earliest consequences of p55 TNF-R ligation is recruitment of the signaling protein TRADD (TNFR1-associated death domain protein) (*25*). We therefore examined whether UV-B exposure also led to TRADD recruitment. The amount of TRADD that coprecipitated with p55 TNF-R after UV-B irradiation was similar to the amount of TRADD that associated with this receptor after incubation with TNF (Fig. 3A).

Both exposure to UV-B light and osmotic stress stimulated tyrosine phosphorylation of EGF-R to nearly the same extent as EGF itself (Fig. 3B). Incubation of cells with TNF weakly stimulated EGF-R tyrosine phosphorylation, but IL-1 had no such effect. If the observed tyrosine phosphorylation reflects EGF-R activation, it should lead to recruitment of adaptor proteins such as growth factor receptor–bound protein 2 (GRB2) and Shc (*19*). Indeed, both UV-B irradiation and osmotic stress increased the amounts of GRB2 (*20*) and Shc (Fig. 3C) associated with EGF-R.

UV-C and UV-B light and osmotic shock are extremely potent activators of JNKs (*8–10, 17*). If simultaneous activation of all three receptors, and possibly others, is the mechanism by which UV light and osmotic shock activate JNK, treatment of cells with all three ligands should result in strong JNK activation. Indeed, EGF synergized with either IL-1 or TNF to cause more

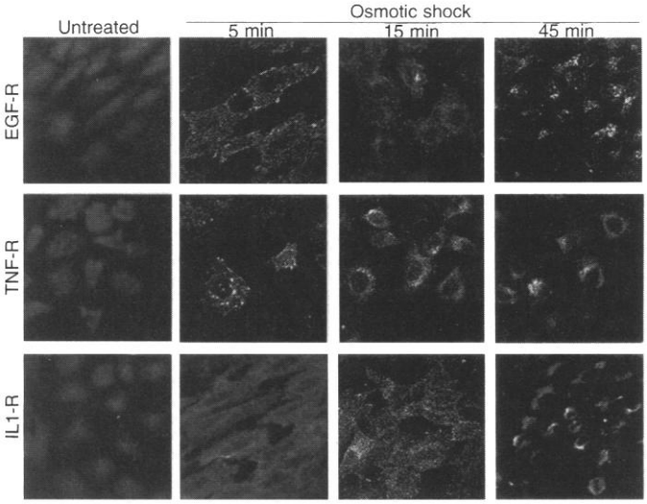

Fig. 2. Effect of osmotic shock on the aggregation state of EGF, IL-1, and TNF receptors. HeLa cells cultured on glass cover slips were processed as described in Fig. 1, except that they were incubated in DMEM containing 600 mM sorbitol (765 mosmol) for the indicated times before fixation.

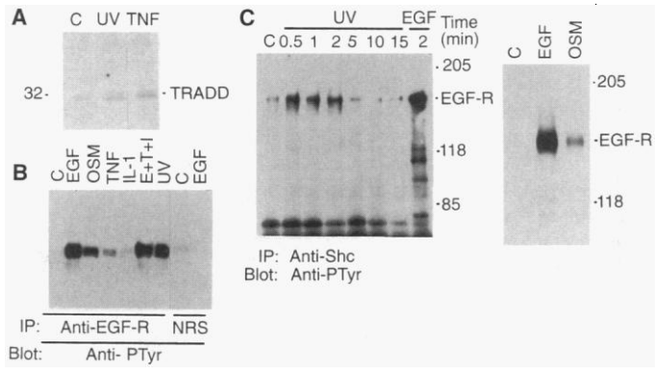

Fig. 3. Activation of the TNF and EGF receptors. (**A**) Association of TRADD with p55 TNF-R after UV-B irradiation. HeLa cells grown on 100-mm dishes were deprived of serum for 36 hours and then left untreated (lane labeled C), incubated with TNF (10 ng/ml), or exposed to UV-B irradiation. After 20 min, cells were incubated with the cleavable cross-linker DTSSP for 10 min at 4°C. The reaction was stopped by incubation in phosphate-buffered saline containing 10 mM ammonium acetate for 5 min at 4°C. Cells were then lysed, and lysate proteins (1 mg) were immunoprecipitated with antibody to p55 TNF-R (AB225-PB), separated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membrane, and probed with antibodies to TRADD as described (*25*). The position of 32-kD molecular size marker is indicated. (**B**) Stimulation of EGF-R tyrosine phosphorylation. HeLa cells grown on 100-mm dishes were deprived of serum for 36 hours and then left untreated (C), incubated with EGF (100 ng/ml), high-osmolarity medium (OSM; DMEM + 600 mM sorbitol), TNF (10 ng/ml), IL-1α (4 ng/ml), a combination of EGF, TNF, and IL-1α (E+T+I), or exposed to UV-B irradiation (600 J·m^{-2}). After 20 min cells were collected and lysed and proteins were immunoprecipitated (IP) with a polyclonal rabbit antiserum to EGF-R (RK2) (anti-EGFR) or normal rabbit serum (NRS) (*8*), separated by SDS-PAGE, and transferred to a PVDF membrane and immunoblotted with a monoclonal antibody to phosphotyrosine (anti-PTyr) (PY20, Transduction Laboratories, Lexington, Kentucky). Antibody-antigen complexes were detected by chemiluminescence with an ECL kit (Amersham Life Sciences). The molecular size of the detected band is 170 kD, the size of an EGF-R monomer. (**C**) Induction of Shc binding. Serum-deprived HeLa cells were left untreated (C), irradiated with UV-B light, or incubated with EGF or high-osmolarity medium (OSM). At the indicated times or after 20 min (right hand panel), the cells were collected and lysed as described. Samples (200 μg of protein) were immunoprecipitated with a polyclonal rabbit antibody to Shc (number 313) and analyzed by immunoblotting, as described above, with anti-PTyr. The migration position of a band identical in size to the EGF-R is indicated. The same band was also detected by probing the blot with an antibody to EGF-R (mAB108) (*20*).

activation of JNK (Fig. 4A). Further enhancement was seen when all three ligands were combined, and in that case, the extent of JNK activation was similar to that after UV-B irradiation or osmotic shock. When the activity of another MAPK, extracellular signal-regulated kinase (ERK), was examined, no synergy between EGF, IL-1, and TNF was observed (Fig. 4B). Thus, by combining EGF, IL-1, and TNF, it was possible to generate the same profile of MAPK activation seen after exposure to physical stress: a large increase in JNK activity and only a small increase in ERK activity.

To test whether receptor clustering is responsible for JNK activation by either UV irradiation or osmotic shock, we examined the effect of incubation at lower temperatures. Incubation of cells at 10°C, which is well below the transition temperature of the membrane, prevented receptor clustering after UV-B irradiation or osmotic stress (*20*) and also blocked JNK activation (Fig. 5A). To further substantiate the roles of the various receptors in the UV-B light and osmotic stress responses, we induced receptor down-regulation by incubating the cells with a mixture of EGF, IL-1, and TNF for several hours. This resulted in complete loss of the response to all three ligands and responses to either UV-B light or osmotic stress that were one-fourth to one-seventh those in control cells (Fig. 5B). The residual response may be mediated by other receptors; it is unlikely that the three receptors we tested are the only receptors whose aggregation is induced by these physical agents. Similar results were obtained by incubating the cells with neutralizing antibodies to IL1-R and p55 TNF-R (Fig. 5C). No inhibition of the UV response was found when the cells were incubated with antibodies to IL-1 and TNF (Fig. 5D).

Collectively, these results strongly suggest that the initial signaling event that activates the JNK cascade in response to either UV irradiation (UV-B or UV-C light) or osmotic stress is multimerization and clustering of cell surface receptors for growth factors and cytokines. We predict that any receptor whose activation mechanism involves multimerization (*19*) should be activatable by UV light and osmotic stress. Exactly how two different physical agents, UV light and osmotic stress, lead to multimerization of cell surface receptors is not clear, but physical perturbation of the plasma membrane or a conformational change caused by energy absorption may cause receptor clustering (*24*). Genetic dissection of the osmotic shock response in yeast leading to activation of the MAPK Hog1p revealed the involvement of two osmosensors, Sln1p and Sho1p (*26*). Although homologous proteins may exist in vertebrates, so far none have been reported.

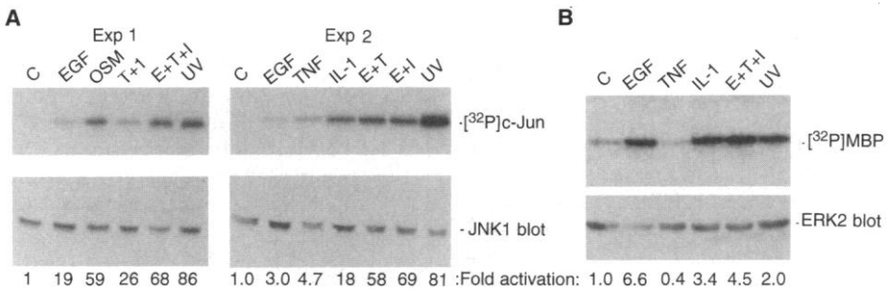

Fig. 4. Stimulation of JNK and ERK activities. (**A**) HeLa cells were deprived of serum for 36 hours and then left untreated (C), or incubated with EGF (E), TNF (T), and IL-1 (I) either alone or in combination, as indicated, or exposed to high-osmolarity medium (OSM) or UV-B irradiation. After 20 min, the cells were collected and lysed, and protein samples (80 μg) were used to measure JNK activity by the binding kinase assay with glutathione-S-transferase fusion protein GST–c-Jun(1–79) as a ligand and substrate (*11*). Samples of extract protein (40 μg) were analyzed by immunoblotting with polyclonal antiserum to JNK1 (*11*). Shown are the results of two experiments in which the kinase activity was quantitated with a phosphoimager and normalized relative to the JNK1 amount in each sample. Similar results were obtained in two other experiments. (**B**) Cells were treated as in (A). Samples of extract protein (100 μg) were immunoprecipitated with a rabbit polyclonal antibody to ERK2 (C-14, Santa Cruz Biotech, Santa Cruz, California) and subjected to immunecomplex kinase assays with myelin basic protein (MBP) as a substrate (*17*). Samples of extract protein (40 μg) were analyzed by immunoblotting with antibodies to ERK2. Kinase activity was quantitated and normalized as described above. Similar results were seen in two experiments.

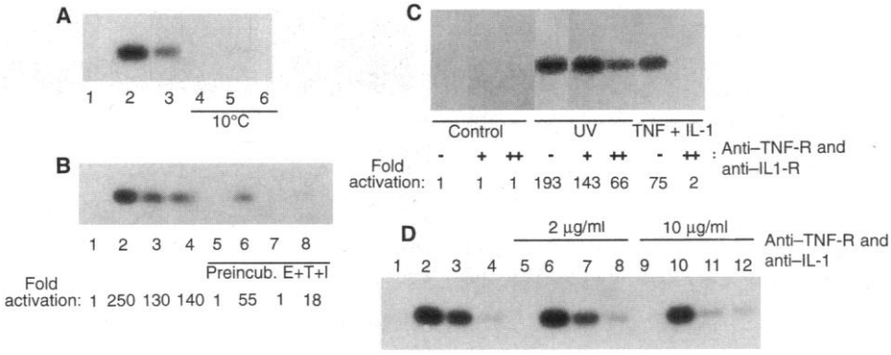

Fig. 5. Inhibition of the UV light and osmotic stress responses by receptor down-regulation or prevention of clustering. (**A**) Inhibition of JNK activation at low temperature. HeLa cells grown on 100-mm culture plates were deprived of serum for 36 hours then incubated at 10°C (lanes 4 through 6) or left at 37°C for 30 min (lanes 1 through 3). The cells were left untreated (lanes 1 and 4) or treated with UV-B light (lanes 2 and 5) or 600 mM sorbitol (lanes 3 and 6). Cells were lysed and extracts were prepared 20 min later for JNK assay as described (Fig. 4). Immunofluorescence analysis indicated that the incubation at 10°C prevented receptor clustering (*20*). (**B**) Inhibition of UV light and osmotic shock responses after receptor down-regulation. HeLa cells were serum deprived and then left untreated (lanes 1 and 5), UV-irradiated (lanes 2 and 6), treated with a combination of EGF, TNF, and IL-α (lanes 3 and 7), or incubated in hyperosmotic medium (lanes 4 and 8). Parallel cultures received the same treatments after incubation with a combination of EGF (100 ng/ml), TNF (10 ng/ml), and IL-α (4 ng/ml) for 4 hours (Preincub. E+T+I) (lanes 5 through 8). After 20 min the cells were collected and lysed, and protein samples (80 μg) were used to measure JNK activity by the binding kinase assay with GST–c-Jun(1–79) as a ligand and substrate. Fold activation of JNK relative to the untreated sample are indicated and was normalized to the amount of JNK assayed as measured by protein immunoblotting. Note that basal JNK activity in E+T+I preincubated cells was twice that in non-stimulated cells (55 cpm compared with 26 cpm). (**C**) Inhibition of signaling induced by UV irradiation with neutralizing antibodies to receptors. HeLa cells were serum deprived and then incubated or not with neutralizing antibodies [5 μg/ml (+) or 20 μg/ml (++)] to the p55 TNF-R and IL1-R for 45 min (MAB225 to p55 TNF-R and AB280-NA to IL1-R, R&D Systems). Cells were then left untreated, UV-irradiated, or treated with a combination of TNF (10 ng/ml) and IL-1α (4 ng/ml). After 20 min the cells were processed as above. Fold activation of JNK normalized to the amount of JNK determined by protein immunoblot are indicated below. (**D**) Ligand neutralization does not interfere with signaling induced by UV light. HeLa cells were either incubated or not with 2 μg/ml or 10 μg/ml concentrations of each antibody to TNF and IL-1α (AB210-NA and AB200-NA, respectively, R&D Systems) for 45 min, then either left untreated (lanes 1, 5, and 9), or treated with UV-B light (lanes 2, 6, and 10) or a combination of EGF, TNF, and IL-1α (lanes 3, 7, and 11) as in Fig. 4. Lanes 4, 8, and 12 were treated with EGF alone. Extracts were prepared and JNK activity was assayed as above.

The amount of UV-B light used in our experiments did not exceed twice the average minimal erythema dose, an amount of UV-B radiation that causes barely detectable skin reddening and is equivalent to 2 to 3 min of solar irradiation on a summer day (*27*). Prolonged sunlight exposure results in fever and a local inflammation. This response may result from activation of JNK and related MAPKs, leading to stimulation of AP-1 activity and cytokine production (*23*). Prolonged exposure to UV-B light causes skin wrinkling, another effect that was attributed to AP-1 activation (*27*). Osmotic stress also induces inflammatory responses in humans similar to the ones induced by IL-1 (*28*). Our findings provide a mechanistic explanation to these observations as well.

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18. UV-B light caused activation of JNK and NF-κB in HeLa, HepG2, PC12, NIH 3T3, and A431 cells. The half-maximal dose for activation of either JNK or NF-κB in HeLa cells was 300 $J \cdot m^{-2}$, whereas the maximal response was observed at 1200 $J \cdot m^{-2}$. The peak emission for our UV-B lamp is 307 to 312 nm, and less than 5% of the emission is at the UV-C range.
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20. C. Rosette, unpublished results. A full-time course study of the effect of EGF on receptor aggregation was done. The 15-min time point is shown in Fig. 1. EGF-R dimerization after treatment of HeLa cells with either EGF or UV-B light was examined by incubating cells with the bifunctional cross-linker DTSSP [3,3′-dithiobis (sulfosuccinimidyl propionate)] before extraction in lysis buffer. Proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by immunoblotting with antibody to EGF-R (mAB108).
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24. Even the initial mechanism of ligand-induced receptor clustering and aggregation is not clear. It is thought that a slight conformational change produced by ligand binding increases the propensity of cell surface receptors to aggregate and cluster within coated pits or caveolae. Most signaling by activated receptors may occur within such structures [R. G. W. Anderson, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 10909 (1993)]. Absorption of UV energy by either membrane proteins or lipids may lead to similar changes in protein conformation and distribution. The changes in membrane tension after osmotic stress may produce similar effects.
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Mapping of a Gene for Parkinson's Disease to Chromosome 4q21–q23

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Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease, affecting approximately 1 percent of the population over age 50. Recent studies have confirmed significant familial aggregation of PD and a large number of large multicase families have been documented. Genetic markers on chromosome 4q21–q23 were found to be linked to the PD phenotype in a large kindred with autosomal dominant PD, with a $Z_{max} = 6.00$ for marker *D4S2380*. This finding will facilitate identification of the gene and research on the pathogenesis of PD.

The first clear description of the common neurodegenerative disorder designated as Parkinson's disease (PD) was provided by James Parkinson in 1817 (*1*). Clinical manifestations include resting tremor, muscular rigidity, bradykinesia, and postural instability. Additional features are characteristic postural abnormalities, dysautonomia, dystonic cramps, and dementia. The specific pattern of neuronal degeneration is accompanied by eosinophilic intracytoplasmic inclusions known as Lewy bodies in surviving neurons in the substantia nigra, locus ceruleus, nucleus basalis, cranial nerve motor nuclei, central and peripheral divisions of the autonomic nervous system, hypothalamus, and cerebral cortex (*2*).

Parkinson's successors speculated on the relative roles of environment and heredity. Gowers in 1880 noted that 15% of his PD patients had affected relatives (*3*). Modern studies of familial aggregation in PD along with the observation of an autosomal dominant pattern of inheritance seen in multicase families have indicated that genetic factors play a substantial etiologic role (*4*). The availability of well-documented families provides an opportunity to carry out DNA linkage studies (*5*).

The discovery of the parkinsonogenic effects of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) led many investigators to search for an environmental factor in the etiology of PD (*6*). The highly selective destruction of the dopaminergic neurons of the substantia nigra caused by MPP^+, the toxic metabolite of MPTP, through inhibition of complex I of the mitochondrial respiratory chain provided a model that explained how an exogenous agent could induce PD (*7*). However, MPTP-induced parkinsonism did not replicate either the clinical course or the pathology of PD. Moreover, subsequent studies did not establish a direct link between PD and mutations in mitochondrial DNA (*8*) or

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