in brain (*19*). We raised antipeptide antibodies to FAK+ (serum SL42) and demonstrated their specificity in transfected COS-7 cells (Fig. 4B). We found that FAK+ was expressed as a protein that was particularly enriched in brain compared with other tissues (Fig. 4C), and in neurons in culture, in which it represented more than 80% of the total FAK (*11*). Phosphorylation of FAK+ was strongly stimulated by anandamide and AA in hippocampal slices and accounted for all the changes in total FAK phosphorylation detected in this preparation (Fig. 4D).

Thus, stimulation of cannabinoid CB1 receptors increased tyrosine phosphorylation of several proteins including FAK+ by inhibiting adenylyl cyclase and PKA. The mechanism by which PKA regulates tyrosine phosphorylation is not known; it may involve inhibition of a tyrosine kinase or, as proposed in *Aplysia* neurons (*20*), stimulation of a tyrosine phosphatase. The effects of anandamide differed from those of AA and from those of neurotransmitters, including norepinephrine, acetylcholine, and glutamate (*7*, *8*), which involve PKC. Therefore, neurotransmitters that raise cAMP concentrations may have an inhibitory effect on protein tyrosine phosphorylation, whereas those that decrease cAMP or activate PKC may have a stimulatory effect. Regulation of FAK+ tyrosine phosphorylation may be an important step in the regulation of ion channels and receptors that are phosphorylated on tyrosine (*21*), as well as of signaling cascades controlling neuronal differentiation and survival (*22*). Thus, phosphorylation of FAK+ provides a common mechanism by which anandamide, AA, or neurotransmitters may exert effects on synaptic plasticity and neuronal trophicity.

REFERENCES AND NOTES

1. M. D. Schaller, J. T. Parsons, *Curr. Opin. Cell Biol.* **6**, 705 (1994); M. A. Schwartz, M. D. Schaller, M. H. Ginsberg, *Annu. Rev. Cell. Dev. Biol.* **11**, 549 (1995).
2. D. D. Schlaepfer, S. K. Hanks, T. Hunter, P. Van der Geer, *Nature* **372**, 786 (1994); H.-C. Chen and J.-L. Guan, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 10148 (1994); C. Guinebault *et al.*, *J. Cell Biol.* **129**, 831 (1995).
3. F. Burgaya, A. Menegon, M. Menegoz, F. Valtorta, J.-A. Girault, *Eur. J. Neurosci.* **7**, 1810 (1995).
4. S. G. N. Grant, K. A. Karl, M. A. Kiebler, E. R. Kandel, *Genes Dev.* **9**, 1909 (1995).
5. S. G. N. Grant *et al.*, *Science* **258**, 1903 (1992).
6. T. J. O'Dell, E. R. Kandel, S. G. N. Grant, *Nature* **353**, 558 (1991); A. R. Boxall, B. Lancaster, J. Garthwaite, *Neuron* **16**, 805 (1996).
7. X.-Y. Huang, A. D. Morielli, E. G. Peralta, *Cell* **75**, 1145 (1993).
8. J. C. Siciliano, M. Gelman, J.-A. Girault, *J. Neurochem.* **62**, 950 (1994).
9. W. A. Devane *et al.*, *Science* **258**, 1946 (1992); V. Di Marzo *et al.*, *Nature* **372**, 686 (1994).
10. L. A. Matsuda, S. J. Lolait, M. J. Brownstein, A. C. Young, T. I. Bonner, *Nature* **346**, 561 (1990).
11. P. Derkinderen, M. Toutant, M. Gelman, J.-A. Girault, unpublished observations.
12. A. C. Howlett, *Annu. Rev. Pharmacol. Toxicol.* **35**, 607 (1995).
13. M. Rinaldi-Carmona *et al.*, *FEBS Lett.* **350**, 240 (1994).
14. F. Desarnaud, H. Cadas, D. Piomelli, *J. Biol. Chem.* **270**, 6030 (1995).
15. M. Wartmann, D. Campbell, A. Subramanian, S. H. Burstein, R. J. Davis, *FEBS Lett.* **359**, 133 (1995).
16. B. Chamak, A. Fellous, J. Glowinski, A. Prochiantz, *J. Neurosci.* **7**, 3163 (1987).
17. S. Lev *et al.*, *Nature* **376**, 737 (1995); H. Sasaki *et al.*, *J. Biol. Chem.* **270**, 21206 (1995).
18. M. D. Schaller, C. A. Borgman, J. T. Parsons, *Mol. Cell. Biol.* **13**, 785 (1993).
19. F. Burgaya and J.-A. Girault, *Mol. Brain Res.* **37**, 63 (1996).
20. G. F. Wilson and L. K. Kaczmarek, *Nature* **366**, 433 (1993).
21. J. F. Hopfield, D. W. Tank, P. Greengard, R. L. Huganir, *ibid.* **336**, 677 (1988); Y. T. Wang and M. W. Salter, *ibid.* **369**, 233 (1994); S. J. Moss, G. H. Gorrie, A. Amato, T. G. Smart, *ibid.* **377**, 344 (1995); M. Menegoz, L. F. Lau, D. Hervé, R. L. Huganir, J.-A. Girault, *Neuroreport* **7**, 125 (1995).
22. S. Finkbeiner and M. E. Greenberg, *Neuron* **16**, 233 (1996).
23. Rat hippocampal slices (300 μm) were prepared from male Sprague-Dawley rats (100 to 150 g) as described (*8*) and incubated (three slices per tube) at 35°C for 50 min before pharmacological treatments. At the end of the experiment, slices were sonicated in a solution of SDS (200 μl, 1% w/v) and sodium orthovanadate (1 mM) in water at 100°C, placed in a boiling water bath for 5 min, and stored at −20°C until biochemical analysis.
24. Neurons, prepared from 17-day-old rat embryos, were cultured in a monolayer in serum-free conditions (*16*). Thirty minutes before drug application, the medium was replaced with artificial cerebrospinal fluid (*8*). After treatment, the fluid was aspirated and the cells were solubilized in 1% (v/v) Triton X-100, deoxycholate (1 mg/ml), SDS (1 mg/ml), 158 mM NaCl, 10 mM tris-HCl (pH 7.2), 1 mM sodium orthovanadate, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, and pepstatin A, leupeptin, and trasylol (50 μg/ml each).
25. For immunoprecipitation, six hippocampal slices were homogenized in ice-cold buffer [100 mM NaCl, 50 mM tris-HCl (pH 7.4), 5 mM EDTA, and 50 mM NaF] containing 1 mM Na^+ orthovanadate, 1% (v/v) NP-40, and protease inhibitors (Complete, Boehringer). Immunoprecipitation was carried out with 20 μl of either preimmune serum or rabbit antisera SL38, or SL42 preadsorbed on protein A–Sepharose beads. SL38 was prepared by immunizing a rabbit against the NH_2-terminal fragment of rat FAK [residues 1 to 377 (*19*)] expressed in *Escherichia coli* as a hexahistidine fusion protein. SL42 was raised against a peptide (Fig. 4A) coupled to keyhole limpet hemocyanin.
26. J. D. Hildebrand, M. D. Schaller, J. T. Parsons, *J. Cell Biol.* **123**, 993 (1993).
27. The plasmid used for transfection, pCMV2, was derived from pBK-CMV (Stratagene) by deletion of an Nhe I–Sal I fragment corresponding to the bacterial promoter. pCMV2-C^+19 and pCMV2-C^-19 contained inserts corresponding to rat FAK cDNA with (FAK+) or without (FAK) the insertion coding for the three amino acids Pro-Trp-Arg (*19*). COS-7 cells were transfected in the presence of *N*-[1-(2,3-dioleoyl)propyl]-*N*,*N*,*N*-trimethylammonium methyl sulfate (DOTAP, Boehringer) according to the manufacturer's instructions. Cells were lysed 72 hours after transfection.

28. C. Giaume and J. Glowinski are gratefully acknowledged for critical reading of the manuscript. F.B. was supported by the European Commission Human Capital and Mobility Programme (CT940705), J.C.S. by ECOS (Comité: Evaluation orientation de la Coopération Scientifique) and the European Commission International Scientific Cooperation (CT940038), V.deF. by INSERM, and M.L.B. by Association Française contre les Myopathies.

6 June 1996; accepted 19 July 1996

Neurogenic Amplification of Immune Complex Inflammation

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The formation of intrapulmonary immune complexes in mice generates a vigorous inflammatory response characterized by microvascular permeability and polymorphonuclear neutrophil influx. Gene-targeted disruption of the substance P receptor (NK-1R) protected the lung from immune complex injury, as did disruption of the C5a anaphylatoxin receptor. Immunoreactive substance P was measurable in fluids lining the lung at time points before neutrophil influx and may thus be involved in an early step in the inflammatory response to immune complexes in the lung.

Immune complexes underlie the inflammatory response seen in a variety of rheumatologic illnesses, including arthritis, vasculitides, and systemic lupus erythematosus (*1*). Antigen-antibody aggregates may be deposited locally and incite edema through enhanced microvascular permeability to plasma proteins as well as elicit exudates of acute inflammatory leukocytes typified by the polymorphonuclear neutrophil (PMN). The mechanisms of injury induced by the immune complex are modeled in experimental animals by the Arthus reaction, in which specific antibody and antigen are passively introduced across a vascular barrier (*2*). Studies on rabbit skin and in mice deficient in complement component C5 implicated complement proteins as crucial participants in the inflammatory response (*3*), a role that has been reinvestigated through the use of mast cell and Fc receptor–deficient mice (*4*). We now use strains of mice deficient in the receptors for sub-

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stance P (NK-1R) and the complement anaphylatoxin C5a (C5aR) to define a mechanism for immune complex–mediated acute lung injury.

Mice deficient in NK-1R and C5aR (*5*) were generated by gene targeting. The NK-1R was cloned as a genomic copy from 129 Sv mice (Fig. 1A). Exon 1 was partially deleted, including the initiating methionine codon, and replaced with a cassette encoding *lacZ* and neomycin resistance. We used J1 embryonic stem (ES) cells containing the targeted allele to achieve germline transmission (*6*) (Fig. 1B). The homozygous NK-1R$^{-/-}$ mice were grossly normal developmentally, were fertile, and appeared to be healthy under barrier isolation conditions. When wild-type and NK-1R$^{-/-}$ animals were challenged with capsaicin [an inflammatory substance derived from hot peppers that induces substance P release from sensory neurons known as C fibers (*7*)], ear edema responses in the NK-1R$^{-/-}$ mice were significantly blunted (*8*) (Fig. 1C). Heterozygote animals developed intermediate responses significantly different from those of NK-1R$^{-/-}$ animals ($P = 0.002$) but not from those of wild-type animals ($P > 0.05$). Immunoreactive NK-1 receptor was not detected in the brains or mucosal organs of NK-1R$^{-/-}$ mice (*9*).

To probe the role of substance P in the immune complex–mediated lung injury, we injected wild-type and NK-1R$^{-/-}$ mice with chicken egg albumin in the tail vein and with polyclonal rabbit antibody to chicken egg albumin intratracheally (*4*, *10*). Evans blue dye was subsequently introduced into the tail vein to gauge endothelial permeability. After 4 hours PMN infiltration into the lung was measured by determining the concentration of the leukocyte enzyme myeloperoxidase (MPO) in lung homogenates (*11*); permeability was determined as the leakage of Evans blue dye recovered with bronchoalveolar lavage (BAL) (*12*). NK-1R$^{-/-}$ mice had near complete protection from the inflammatory response, as assessed histologically (Fig. 2). The lung tissues of immune complex–challenged NK-1R$^{-/-}$ mice appeared to be no different from those of controls challenged with antibody or saline. These observations were quantitated by measuring extravasation of Evans blue dye into the alveolar space and the exudation of neutrophils into the airways and lung alveoli (Fig. 3). These data demonstrate a critical role for release of substance P and interaction with NK-1R in immune complex–mediated injury in the lung.

The critical role for complement in this model of acute lung injury in mice was also shown by challenging the C5aR$^{-/-}$ mice, selectively eliminating contributions of the C5a anaphylatoxin (*3*, *5*). The phenotype of the C5aR$^{-/-}$ mice was identical to that observed for the NK-1R$^{-/-}$ animals (Figs. 2 and 3). Thus, both substance P and C5a, acting through their specific heterotrimeric GTP-binding protein (G protein)–coupled receptors, were essential for the formation of the full injury seen in this model.

We next investigated whether substance P was measurable in the immune complex model. Samples of wild-type BAL fluids were assayed for immunoreactive substance P as well as for tumor necrosis factor-α (TNF-α) (*4*, *10*). Substance P and TNF-α immunoreactivity were at the lower limits of detection in the control (saline-challenged) mice, whereas immune complex–mediated lung injury in wild-type animals

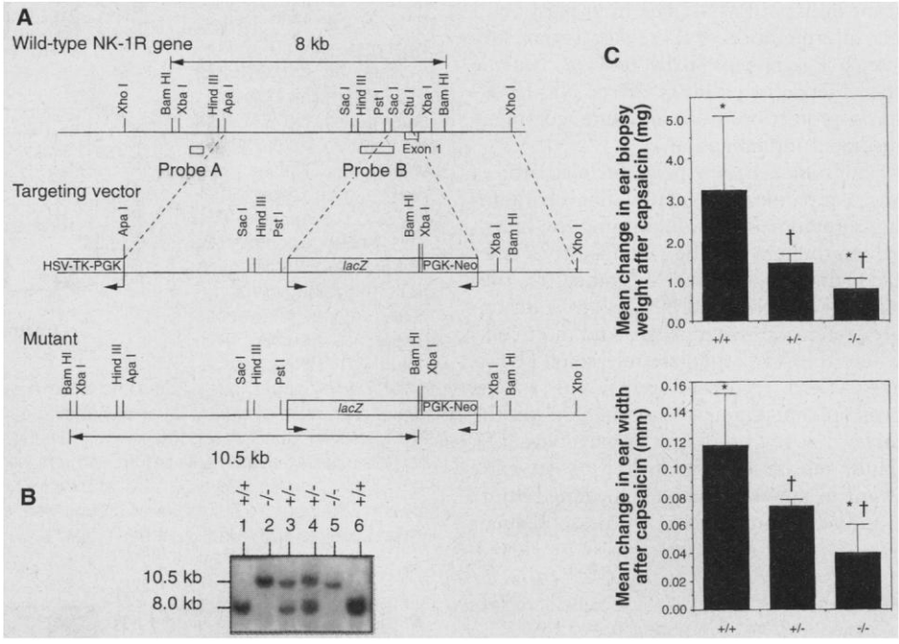

Fig. 1. (**A**) Diagram of wild-type loci, targeting vector, and mutant loci of the mouse NK-1R gene. An ~12-kb Bam HI–Xho I fragment of the NK-1R gene including exon 1 and ~7 kb of the 5′ sequence is shown. Most of exon 1 was deleted and replaced with the genes for β-galactosidase and neomycin resistance. Homologous recombination led to a diagnostic pattern when a probe within the targeted vector (probe B) was used (*6*). (**B**) Southern blot analysis and genotyping of progeny. Genomic DNA was isolated from a litter of pups from one heterozygous breeding pair, subjected to restriction enzyme digestion with Bam HI, and hybridized with probe B. The genotypic analysis of a representative litter is shown. Probe B hybridizes to an 8-kb Bam HI band in the wild-type allele and to a 10.5-kb Bam HI band in the mutant allele. (**C**) Capsaicin-induced ear edema in wild-type (+/+), heterozygous (+/−), and homozygous (−/−) NK-1R littermates. The change in ear biopsy weight (upper panel) or ear width (lower panel) 30 min after capsaicin exposure, compared with the vehicle-exposed contralateral ear, is shown (*8*). (Differences between heterozygous and wild-type responses were not significantly different.) Data represent the means ± SEM of four wild-type, five heterozygous, and six homozygous mice. Upper panel: *$P = 0.01$, †$P = 0.002$; lower panel: *$P = 0.006$, †$P = 0.02$.

Table 1. Substance P and TNF-α in BAL fluid of mice challenged with intrapulmonary immune complexes. Substance P–like immunoreactivity (SPLIR) and TNF-α in BAL fluid were determined with enzyme immunoassay kits according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, Michigan, for substance P; Endogen, Cambridge, Massachusetts, for TNF-α). Concentrations of substance P and TNF-α in wild-type and gene-deleted animals were not significantly different ($P > 0.05$). The PBS control experiments were performed with wild-type animals. Values are means ± SEM; $n = 2$ at each time point for substance P; numbers of animals are as indicated for TNF-α. Immune complex exposure to macrophages elicited baseline SPLIR at 4 hours. ND, not determined.

Genotype	SPLIR (pg/ml)			TNF-α (ng/ml)
	1 hour	2 hours	4 hours	4 hours
Wild-type	275 ± 78	418 ± 81	134 ± 23	2.8 ± 0.8 ($n = 6$)
NK-1R$^{-/-}$	ND	ND	ND	3.7 ± 0.7 ($n = 9$)
C5aR$^{-/-}$	366 ± 16	355 ± 153	153 ± 66	1.8 ± 0.5 ($n = 8$)
PBS control	12	14	10 ± 4	0.05 ± 0.01 ($n = 3$)
Immune complex + macrophages	ND	ND	20	ND

was associated with measurable substance P and TNF-α (Table 1). When these mediators were assayed in the immune complex–challenged, gene-targeted mice, substance P and TNF-α were detected at concentrations similar to those found in immune complex–challenged, wild-type animals, even though the gene-targeted strains had no inflammation. Further, substance P was detected at as early as 1 hour in BAL fluids, before significant influx of PMNs. The most parsimonious interpretation of these data is that substance P acts proximally to C5a, because the absence of either C5aR or NK-1R affords protection from immune complex–mediated inflammation.

Substance P is a powerful mediator of vascular permeability, and C-fibers containing substance P are found lining the mucosal epithelium of the aerodigestive tract (*13*). Immunoreactive substance P, substance P mRNA, and NK-1R are additionaly present on macrophages and mast cells, where they can stimulate release of TNF-α (*14*). NK-1 receptors are present on the postcapillary venular endothelium of the airway but not on that of the alveolus (*15*). Thus, release of substance P as an early event in immune complex–mediated injury could act as an amplifier of the subsequent inflammatory changes. Small quantities of immune complexes formed along the airway surface may release local substance P. Tachykinin acts at the mucosal microvasculature to cause leakage of plasma proteins, supplying more antigen for the uncom-

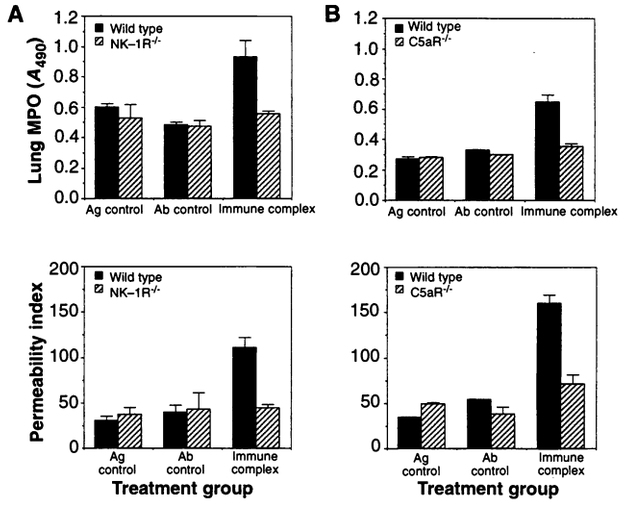

Fig. 3. (**A**) MPO accumulation in lung (upper panel) and BAL fluid permeability index (lower panel) 4 hours after intrapulmonary deposition of IgG immune complexes in NK-1R$^{-/-}$ or wild-type (NK-1R$^{+/+}$ or NK-1R$^{+/-}$) mice. Upper panel: $^*P < 0.02$; lower panel: $^*P < 0.001$. (**B**) MPO accumulation in lung (upper panel) and BAL fluid permeability index (lower panel) in C5aR$^{-/-}$ or C5aR$^{+/+}$ mice. Upper panel: $^*P < 0.003$; lower panel $^*P < 0.001$. Antibody controls (Ab control) received antibody to chicken egg albumin intratracheally without intravenous injection of albumin. Mice treated with PBS intratracheally followed by intravenous chicken egg albumin served as antigen controls (Ag control). The permeability index is a measure of extravasation of protein-bound Evans blue dye into the lung. Values are means ± SEM for seven C5aR$^{-/-}$ and seven wild-type mice and for 10 NK-1R$^{-/-}$ and 12 heterozygote and wild-type mice. Measurements of MPO accumulation after immune complex challenge are the means for five C5aR$^{-/-}$ and five wild-type mice, and for four NK-1R$^{-/-}$ and four wild-type animals. Measurements between wild-type and homozygous groups were considered to be statistically significant by comparison with an unpaired two-tailed Student's *t* test.

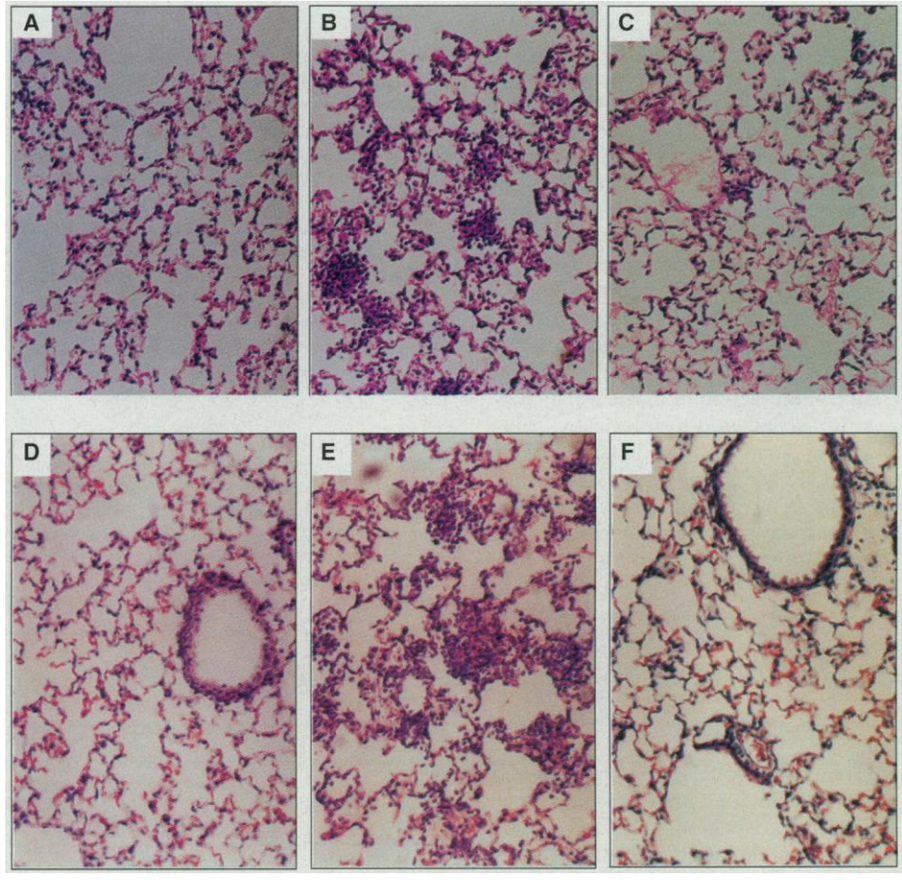

Fig. 2. Histological analysis of immune complex–mediated lung injury in wild-type and NK-1R$^{-/-}$ (upper panel) and C5aR$^{-/-}$ (lower panel) mice. (**A**) Control NK-1R$^{+/+}$ mouse lung 4 hours after intratracheal injection of antibody to chicken egg albumin immunoglobulin (Ig) G and intravenous injection of PBS; there is no lung injury. (**B**) NK-1R$^{+/+}$ mouse lung 4 hours after intratracheal injection of antibody to chicken egg albumin IgG and intravenous injection of chicken egg albumin; these treatments usually induced a generalized reaction with neutrophil accumulation, edema, and hemorrhage. (**C**) NK-1R$^{-/-}$ mouse lung 4 hours after intratracheal injection of antibody to chicken egg albumin IgG and intravenous injection of chicken egg albumin; these samples lacked generalized lung injury. (**D**) Control C5aR$^{+/+}$ mouse lung 4 hours after intratracheal injection of antibody to chicken egg albumin IgG and intravenous injection of PBS; there is no injury, similar to that found in the NK-1R$^{+/+}$ mice. (**E**) C5aR$^{+/+}$ mouse lung 4 hours after intratracheal injection of antibody to chicken egg albumin IgG and intravenous injection of chicken egg albumin; these treatments usually induced a generalized reaction with neutrophil accumulation, edema, and hemorrhage. (**F**) C5aR$^{-/-}$ mouse lung 4 hours after intratracheal injection of antibody to chicken egg albumin IgG and intravenous injection of chicken egg albumin; these samples lacked generalized lung injury. All sections were stained with hematoxylin and eosin and analyzed with the investigator blinded to genotype; original magnification: ×200. Results are representative of 36 wild-type and homozygous mice.

plexed antibody already deposited in the airway, and thus amplifying the response. Additionally, the plasma leakage delivers a local source of the complement components, which become fixed by the immune complexes to ultimately generate C5a. The amplification of the formation of immune complexes, as well as the delivery of a local source of complement, occurs through substance P acting at the microvasculature of the airway mucosa.

The present results thus extend the previous model of the inflammatory cascade in immune complex–mediated injury (*4*). In that model, Fc receptor engagement in the skin was shown to be a critical initiating event in the production of edema and neutrophil exudation. However, the identity of the mediators that could link the inciting (Fc) stimulus with the subsequent edema and PMN influx remained conjectural (*16*). Our data provide a plausible linkage between the Fc stimulus and neurohumoral mediators by showing that the complement-dependent inflammatory response is C5aR-dependent and is downstream from the immune complex release of substance P, which amplifies the inflammatory response. The present data heighten interest in investigating the tachykinins as being critical in other inflammatory models such as inflammatory bowel disease and asthma.

REFERENCES AND NOTES

1. K. A. Davies, J. A. Schifferli, M. J. Walport, *Springer Semin. Immunopathol.* **15**, 397 (1994).
2. M. Arthus, *C. R. Seances Soc. Biol. Paris* **55**, 817 (1903).
3. C. G. Cochrane and A. Janoff, in *The Inflammatory Process*, B. W. Zweifach, L. Grant, R. T. McCluskey, Eds. (Academic Press, New York, 1974), vol. 3, chap. 3; B. F. Ramos, Y. Zhang, B. A. Jakschik, *J. Immunol.* **152**, 1380 (1994); G. L. Larsen, B. C. Mitchell, P. M. Henson, *Am. Rev. Respir. Dis.* **123**, 434 (1981).
4. Y. Zhang, B. F. Ramos, B. A. Jakschik, *Science* **258**, 1957 (1992); B. F. Ramos, R. Qureshi, K. M. Olsen, B. A. Jakschik, *J. Immunol.* **145**, 1868 (1990); Y. Zhang, B. F. Ramos, B. A. Jakschik, *J. Clin. Invest.* **88**, 841 (1991); B. F. Ramos, Y. Zhang, B. A. Jakschik, *Eur. J. Immunol.* **22**, 2381 (1992); D. L. Sylvestre and J. V. Ravetch, *Science* **265**, 1095 (1994).
5. U. E. Höpken, B. Lu, N. P. Gerard, C. Gerard, *Nature*, in press.
6. An ~12-kb Bam HI–Xho I fragment of the NK-1R gene, including exon 1 and ~7 kb of the 5′ sequence, was cloned from a mouse Sv 129 genomic library. Through sequential cloning steps, most of exon 1 was deleted, including the sequence from the Sac I site ~80 nucleotides downstream from the TATAA sequence to the Stu I site just before the end of the exon 1 coding sequence, and replaced with the genes for β-galactosidase and neomycin resistance. The construct was transfected into J1 embryonic stem cells and doubly selected for integration with G418 and gancyclovir. Homologous recombination was determined by restriction analyses of independent clones, with both flanking and internal probes (probes A and B, respectively). Three clones among ~100 screened were positive for homologous recombination into the NK-1R locus. They were injected into mouse blastocysts and resulted in eight chimeric offspring, which were bred with C57BL/6 mice for germline transmission.
7. P. Holzer, *Neuroscience* **24**, 739 (1988).
8. Littermate mice of ~30 g were used. Capsaicin was dissolved in acetone (10 mg/ml) and 25 μl was applied topically to both surfaces of one ear. Acetone alone was applied to the contralateral ear. After 30 min, ear thickness was measured with dial calipers, and 6-mm punch biopsies were weighed [H. Inoue, N. Nagata, Y. Koshihara, *Br. J. Pharmacol.* **110**, 1614 (1993); C. R. Mantione and R. Rodriguez, *ibid.* **99**, 516 (1990)].
9. N. Bunnett, personal communication.
10. Mice matched for sex and age (12 to 14 weeks) were used for studies of immune complex–induced lung injury [J. S. Warren *et al.*, *J. Clin. Invest.* **84**, 1873 (1989)]. Rabbit antibody to chicken egg albumin (300 μg) was injected intratracheally, followed by injection of chicken egg albumin (20 mg/kg) into a tail vein. Some animals were tracheostomized after they were killed, and BAL was performed with saline. The recovered BAL fluid was assessed for differential cell counts and cytokine measurements. Permeability indexes were determined by comparing the amount of Evans blue in BAL fluid with the amount present in 1 ml of plasma. No statistical differences were observed between wild-type animals and heterozygotes in this assay. Paraffin sections were prepared for histologic analyses after inflation of the lung with 10% neutral buffered formalin. All animal studies were performed according to institutional NIH guidelines for animal use and care.
11. P. P. Bradley, D. A. Priebat, R. D. Christensen, G. Rothstein, *J. Invest. Dermatol.* **78**, 206 (1982). Lung was homogenized in phosphate-buffered saline (PBS) and centrifuged at 200*g*; the cell pellet was lysed with 0.2% NaCl and suspended in 2 ml of 0.1 M phosphate buffer, pH 6.8. After two freeze-thaw cycles, the sample was sonicated and centrifuged at 10,000*g*, and the supernatant was assayed.
12. T. L. Buckley and F. P. Nijkamp, *J. Immunol.* **153**, 4169 (1994).
13. J. D. Boomsma and S. I. Said, *Chest* **101**, 389S (1992); J. Barnes, J. N. Baraniuk, M. G. Belvisi, *Am. Rev. Respir. Dis.* **144**, 1187 (1991); S. Mazumdar and K. M. Das, *Am. J. Gastroenterol.* **87**, 176 (1992).
14. D. W. Pascual and K. L. Bost, *Immunology* **71**, 52 (1990); J. Luber-Narod, R. Kage, S. E. Leeman, *J. Immunol.* **152**, 819 (1994); K. L. Bost, S. A. Breeding, D. W. Pascunal, *Reg. Immunol.* **4**, 105 (1992); J. C. Ansel, J. R. Brown, D. G. Payan, M. A. Brown, *J. Immunol.* **150**, 4478 (1993).
15. P. Baluk, N. W. Bunnett, D. M. McDonald, *Am. J. Respir. Crit. Care Med.* **153**, A161 (1996).
16. J. V. Ravetch, *Cell* **78**, 553 (1994).

17. We thank B. Koller for support, advice, encouragement, and blastocyst injections; L. Kobzik for blinded histological analyses; C. De Dios for technical assistance; and M. Soares for help with enzyme-linked immunosorbent assay measurements. Supported by NIH grants HL36162 and HL51366, the Rubenstein-Cable Cystic Fibrosis Research Fund at the Perlmutter Laboratory, the Medical Research Council of Canada (C.R.B.), and Deutsche Forschungsgemeinschaft (U.E.H.).

23 May 1996; accepted 29 July 1996

ER Degradation of a Misfolded Luminal Protein by the Cytosolic Ubiquitin-Proteasome Pathway

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Secretion of proteins is initiated by their uptake into the endoplasmic reticulum (ER), which possesses a proteolytic system able to degrade misfolded and nonassembled proteins. The ER degradation system was studied with yeast mutants defective in the breakdown of a mutated soluble vacuolar protein, carboxypeptidase yscY (CPY*). The ubiquitin-conjugating enzyme Ubc7p participated in the degradation process, which was mediated by the cytosolic 26S proteasome. It is likely that CPY* entered the ER, was glycosylated, and was then transported back out of the ER lumen to the cytoplasmic side of the organelle, where it was conjugated with ubiquitin and degraded.

The ER is the site of entry of proteins into the secretory pathway. Such proteins are translocated in an unfolded state through the membrane of this organelle, and, during the subsequent folding process, N- and O-linked glycosylation occurs and disulfide linkages are formed. The large number of unfolded proteins that enter the lumen of the ER must be protected from aggregation and be maintained in a folding-competent state (*1*). Thus, the ER contains a high concentration of molecular chaperones that promote protein translocation, folding, and oligomerization (*2*). Inefficient folding, unbalanced subunit synthesis, or mutations in secretory proteins result in the translocated polypeptides failing to assume their correct conformation (*3*). The ER can eliminate these proteins through selective degradation associated with a pre-Golgi compartment (*3*, *4*). However, little is known about the mechanisms and proteolytic systems that mediate this ER degradation of proteins.

A mutated plasma membrane protein, the cystic fibrosis transmembrane conductance regulator (CFTR), is retained in the ER membrane and degraded by the ubiquitin-proteasome system (*5*). Two mutated soluble vacuolar proteins of the yeast *Saccharomyces cerevisiae*, carboxypeptidase yscY (CPY*) and proteinase yscA (PrA*), are imported into the lumen of the ER but do not reach the vacuole; instead, they remain within the ER and are degraded (*6*). The absence or mutation of the ER membrane protein Der1p abolishes degradation of CPY* and PrA* in yeast (*7*); CPY* is localized in the ER in cells devoid of Der1p (*7*).

We now describe the *DER2* gene which, when mutated, also results in the accumulation of CPY* and PrA* (*7*, *8*). The *DER2* gene was cloned by complementation of the *der2-1* mutation (*9*). Genetic analysis and

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