Vol. 177, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1991 Pages 8-16

ISOLATION AND CHARACTERISATION OF THE HUMAN LUNG NK-2 RECEPTOR GENE USING RAPID AMPLIFICATION OF cDNA ENDS

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Received April 12, 1991

Functional cDNA clones for human NK-2 receptor were isolated from human lung RNA using a polymerase chain reaction (PCR) based method (RACE-PCR). In this method the cDNA was isolated as 5' end and 3'-end fragments; the entire cDNA was obtained by RNA-PCR. The sequence derived was 398 amino acids in length encoding an open-reading frame that was highly homologous to both the bovine and rat NK-2 receptor. The entire human cDNA sequence was cloned into a mammalian expression vector and mRNA was synthesised by in vitro transcription. Applications of tachykinins caused membrane current responses in Xenopus oocytes injected with the in vitro synthesised mRNA. The most potent of the three tachykinin peptides tested was neurokinin A. We have screened a human cosmid library and isolated a clone which contains the entire NK-2 receptor gene. The gene contains five exons and we have determined the complete sequence of the exons and the intron-exon junctions.

Tachykinins were first recognised for their spasmogenic effect on smooth muscle. Subsequent studies showed that the tachykinins are a family of naturally occurring bioactive neuropeptides which share a common carboxy-terminal amino acid sequence but have differences at their amino termini and it is this difference which determines their receptor affinities (1). The mammalian tachykinin system consists of three distinct peptides, substance P, neurokinin A (substance K) and neurokinin B (neuromedin K). A large number of pharmacological and ligand binding studies provide evidence that there are three different types of tachykinin receptor, termed NK-1, NK-2 and NK-3 receptors (2, 3). Each tachykinin recognises the three receptor types but with varying avidity. The preferred natural agonist for the NK-1 receptor it is substance P, for the NK-2 receptor it is neurokinin A and for the NK-3 receptor it is neurokinin B. In the respiratory system, tachykinins have a number of important physiological actions (4) including vasodilation, mucus secretion and bronchoconstriction. In the lung, NK-2 receptors are believed to be involved in the bronchospastic action

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0006-291X/91 $1.50

of endogenous tachykinins and therefore may play an important role in pulmonary disorders.

The three types of receptor have now been cloned and expressed from rat (5-8), and the bovine NK-2 receptor has also been studied (9). All belong to the family of seven-helix receptors which are coupled to G proteins. This investigation reports the isolation and expression of a cDNA clone for the human NK-2 receptor, as judged by functional expression in oocytes. We also described the molecular organisation of the gene for this receptor.

Materials and Methods

Isolation and characterisation of cDNA. Total RNA was isolated (10) from human lung tissue from a 65 years old male. PCR-based methods (RACE-PCR) (11, 12) were used to isolate the 5'- and 3'-ends of the human NK-2 receptor cDNA as outlined in Figure 1. The 3'-end was obtained as follows: First strand cDNA was synthesised (cDNA kit, Boehringer Corp Ltd) from 5μg of total RNA using the 58-mer R1R2(dT)17 primer (25pmol) 5'd(AAGGATCCGTCGACATCGATAATAC GACTCACTATAAGGGA(T)17). A primary PCR reaction was performed using one amplimer derived from the 5' end of the RACE 58mer R1 = 5'd(AAGGATCCGTCGACATCGATAAT) and a gene specific amplimer, GSA1 = 5'd(ACGGTGACCAACTACTTCATCGTC) which was derived from a consensus sequence in the second transmembrane segment (TMS) of rat and bovine NK-2 receptor (7, 9). PCR (13) was performed at 65°C for 2 min, 72°C for 2 min and 92°C for 2 min for 33 cycles using a Techne PHC-1 thermal cycler and Taq polymerase (Amplitaq) using conditions described by the manufacturers (Perkin Elmer Cetus). The products of this primary reaction (0.1%) were used for a secondary PCR with nested amplimers. One amplimer was a nested RACE amplimer, R2 = 5'd(GATAATACGACTCACTATAAGGGA) and the other a nested gene specific amplimer, GSA2 = 5'd(TGCTGGCATCTGGCTGGTGGCCCTGGC) which was chosen from a region in the fourth TMS. This region is identical between rat and bovine NK-2 receptor but is quite different (especially at the 3' end of the amplimer) in rat substance P (NK-1) receptor and rat neuromedin K (NK-3) receptor. Secondary products were Southern blotted and hybridised to an 'internal' oligonucleotide, GSA3 = 5'd(GTAGGGCAGCCAGCAGATGGCAAA) derived from the sixth TMS of rat and bovine NK-2 receptor. A 1000bp product was identified which was not visible with ethidium bromide staining, however sufficient material for direct sequencing (14) was obtained by further PCR using the same two amplimers.

The 5' end was obtained by similar methods. First strand cDNA was synthesised from 5μg human lung total RNA using a gene specific amplimer GSA3 (25pmol) and the products were purified by spin dialysis (Centricon 100, Amicon Corp). The cDNA was tailed at its 3' end with A's using terminal transferase (Gibco BRL) and second strand cDNA synthesised with the RACE 58mer, R1R2(dT)17 using Taq polymerase Primary PCR was performed using the RACE outer amplimer (R1) and GSA4 = 5'd(AGCACCACCAGCACCATGGTCTTC) which was derived from the sixth TMS of human NK-2 receptor. 0.1% of the primary products were used for secondary PCR with the nested RACE amplimer (R2) and a nested GSA5 = 5'd(GCCAGGGCCACCAGCCAGATGCCAGCA) derived from the fourth TMS this being complementary to GSA2. A product of 750bp was identified by hybridisation with GSA-1 and this was DNA sequenced directly.

Full-length cDNA was obtained by using human amplimers hFL1 = 5'd(CTGATGTGCCACCACGCTTGGCATC) and hFL2 = 5'd(GGACACCACACTCTTTCTAACAACTTG) which are approximately 142bp upstream of the ATG initiation codon and 169bp downstream of the stop codon respectively. First strand cDNA was synthesised from human lung RNA using oligo(dT) as primer and then primary PCR was performed. No visible products (1505bp) were observed with ethidium bromide staining and therefore a secondary PCR was performed with the nested amplimer hFL3 = 5'd(GCGATATCTTGGCATCTGCTCCTCTCCCTG) and hFL4 = 5'd(CCGTCTAGAGTGATGATTC

ACTTCAACTGG). These contained EcoRV and XbaI restriction sites at their 5'ends respectively. The correct sized PCR product (1441bp) was obtained and cloned unidirectionally into pMAMneoBLUE (Clontech). 11 recombinants were totally sequenced and 3/11 contained no PCR errors.

Functional expression of human NK-2 receptor. mRNA was in vitro transcribed using T7 polymerase from a pMAMneoBlue-hNK-2 receptor clone which had been linearised by digestion with SalI and then with XbaI. An in vitro transcription kit (Promega) was used and the mRNA was capped with M7GpppG (Boehringer Corp Ltd). The synthesized mRNA (approximately 5ng of mRNA per oocyte) was microinjected into Xenopus oocytes. Electrophysiological recordings were made at least 24 hours after injection, using standard two electrode voltage clamp techniques (14) with a holding clamp potential of -60mV and stepping by ± 20mV. Substance P and neurokinin B were from Cambridge Research Biochemicals and neurokinin A was from Peninsula Labs.

Isolation and characterisation of genomic clones. A human cosmid library comprising partial Sau3A-digested human DNA in BamHI digested pcos2EMBL was screened using the human NK-2 receptor cDNA as a probe. Positively hybridising colonies were purified and restriction mapped. The DNA sequence of hNK-2 receptor cosmid was determined by either directly sequencing cosmid DNA (16) or by sequencing restriction fragments which had been subcloned into pBluescript (Stratagene, LaJolla) by the dideoxy chain termination method (17) using Sequenase T7 DNA polymerase (USB).

Results

Isolation of human NK-2 receptor cDNA The scheme used to isolate the 5' and 3' ends of the human NK-2 receptor by PCR-based methods is shown in Fig 1. Once the 5' and 3' ends had been identified the complete cDNA was isolated by PCR using amplimers 121bp upstream of the ATG initiation codon and 101bp downstream of the stop codon. Direct sequence analysis of the full-length cDNA has shown that the individual used in this study is heterozygous at amino acid positions 23(ACC or ATC; Thr or Ile) and at position 375(CGT or CAT; Arg or His).

We have cloned and sequenced the PCR product and used a clone which is Thr^{23}, Arg^{375} for further characterisation. The human NK-2 receptor consists of 398 amino acids with a relative molecular mass of 44,425. This protein shares a significant sequence similarity with other seven-helix G-protein coupled receptors and in particular, is highly homologous to the rat and bovine NK-2 receptors (7,9) (86% and 91% respectively) particularly in the seven transmembrane segments.

There are two potential N-glycosylation sites at the amino-terminal region (Asn 11 and Asn 19) whilst the third cytoplasmic loop and the cytoplasmic C-terminal tail have several serine and threonine residues. These residues have the potential to be phosphorylated by serine/threonine protein kinases, thus suggesting that receptor function may be regulated by protein kinases.

Electrophysiological Characterisation of NK-2 receptor The specificity of tachykinin peptide applications on the human NK-2 receptor was studied using functional expression in Xenopus oocytes (Fig 2). Oocytes injected with the

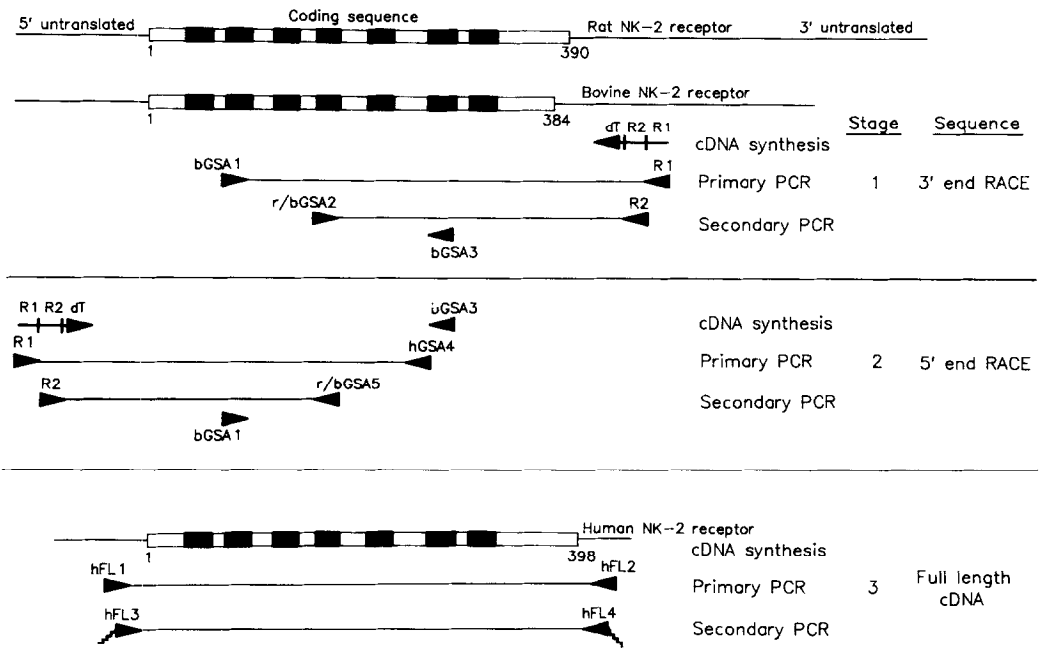

Fig. 1. Isolation of human NK-2 receptor cDNA. Schematic diagram of rat and bovine NK-2 receptor clones and the stages used to isolate human NK-2 receptor cDNA. The priming locations for oligonucleotides used in this study are shown, arrowheads indicate 3' ends, wavy lines non-NK-2 receptor sequences added for cloning. RACE = Rapid Amplification of cDNA ends, GSA = Gene Specific Amplimer, b = bovine sequence, r/b = rat and bovine, h = human. The 5' and the 3' end RACE products were obtained using nested amplimers (see Materials and Methods). Full-length cDNA was then amplified and cloned into pMAMneoBlue.

human NK-2 receptor mRNA developed responses to neurokinin A (marked increases in membrane conductance) within 24 hours after injection, though the size of response is increased further at 48 hours after injection. Clear responses were observed to neurokinin A at concentrations of 10^{-9}M to 10^{-7}M,

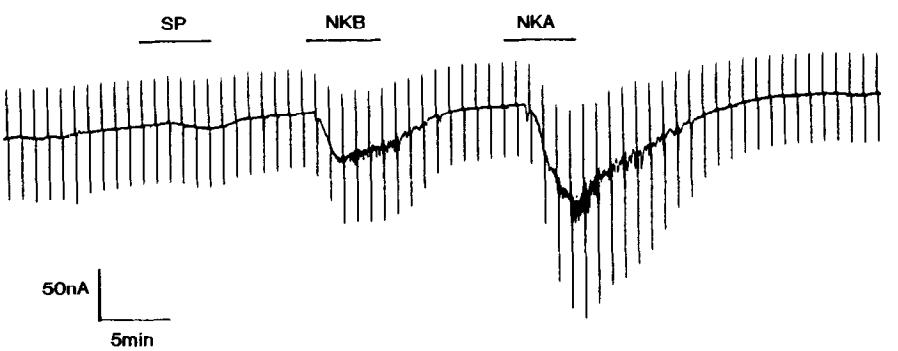

Fig. 2. Current traces recorded from a Xenopus oocyte injected with the *in vitro* synthesised human NK-2 receptor mRNA (approx 5ng). Applications were 10^{-7}M substance P (SP), 10^{-7}M neurokinin B (NKB), and 10^{-8}M neurokinin A (NKA) for the times indicated by the bars. Downward deflections correspond to inward currents and increases in the current steps reflect increases in oocyte conductance.

Table 1. Mean peak currents (nA) ± S.E. evoked by applications of tachykinin peptides to Xenopus oocytes injected with *in vitro* synthesised human NK-2 receptor mRNA

Tachykinin peptide concentration (M)	Responses in nA		
	Neurokinin A	Neurokinin B	Substance P
10^{-9}	85 (n=2)	-	-
10^{-8}	159±18 (n=7)	2.5±2.5 (n=4)	0 (n=1)
10^{-7}	>150* (n=1)	95±59 (n=6)	42±27 (n=6)

* Two other injected oocytes saturated the amplifier with recordings >1.5μA. n = number of experiments.

whereas neurokinin B and substance P induced only small responses at 10^{-8} or 10^{-7}M (Table 1). The rank order of potencies for the three tachykinins was neurokinin A > neurokinin B > substance P, and this order is consistent with that reported for the rat and bovine NK-2 receptor (7, 9). Water injected oocytes were used as controls, and the peptides were applied at the same concentrations as for hNK-2 receptor injected oocytes. A small response was observed to a high concentration of substance P (10^{-6}M); similar responses have been seen by other workers (18). Responses were not observed in water-injected oocytes to any of the peptides at 10^{-7}M, which elicited responses in the NK-2 receptor injected oocytes.

Organisation of the human NK-2 receptor gene A human cosmid library (approximately 200,000 recombinant cosmids) was screened with the human NK-2 receptor cDNA sequence. Positively hybridising colonies were purified and characterised. All of the clones had overlapping restriction patterns and were presumably from the same locus. We have characterised one of these cosmid clones (pHNK2R.11G1) in more detail (Figs 2 and 3). The sizes of the

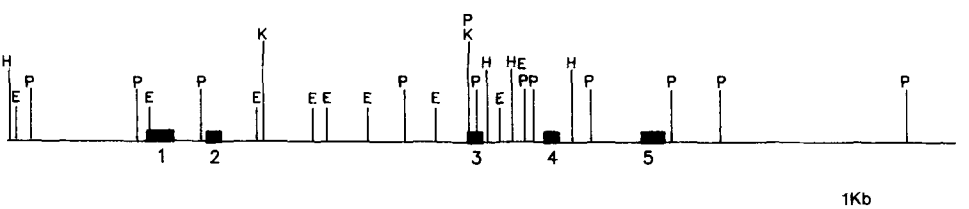

Fig.3. Structure of the human NK-2 receptor gene. The restriction endonuclease map was deduced from both direct cosmid sequencing and from cleavage of the cosmid DNA (pHNK2R.11G1) with EcoRI(E), PstI(P), HindIII(H) and KpnI(K) and hybridisation with oligonucleotide probes. The five exons are numbered and are represented by solid boxes, and the intron sizes are shown.

introns were determined by PCR across the introns and by restriction mapping. The human NK-2 receptor gene spans approximately 17kb and includes 5 exons. The exon-intron junctions were identified by comparing the genomic sequence to the cDNA sequence obtained by RACE-PCR. The splice junctions in this gene (except for intron 1) contained the expected GT splice donor and AG splice acceptor and conform to the consensus sequences established for intronic donor and acceptor splice signals (19, 20).

Interestingly, all the intron/exon junctions correspond to junctions between loops and transmembrane segments in the the amino acid sequence and none of the membrane spanning regions are interrupted by introns. Two transcriptional control elements are found at the 5' end of the hNK-2 receptor gene. The TATA (ATTTATAA) is located between 300 and 307 bases upstream of the initiation codon methionine. There is also a sequence (GGGCTGGTCCCG) which occurs at -356 to -366 and is like a GC box.

Comparison of the 5' flanking sequences of human and bovine NK-2 receptor revealed a sequence homology of 65% between -447 to -156 of human with 1-277 of bovine sequence. This may indicate that expression of the gene may be controlled by regulatory elements within the homologous regions.

An in-frame dipeptide (double underlined) is found 84 bases upstream of the putative initiation codon. Such a small open reading frame in the 5' flanking sequence has been found in hamster $\beta 2$-adrenergic receptor (21), rat D_2 dopamine receptor (22) and guinea-pig platelet activating factor cDNAs (23) (Fig. 4).

Exon 1 contains the 5' untranslated sequence and the first 392bp of the coding sequence. Exon 5 contains the last 256bp of the coding sequence, the termination codon (TGA) and the 3' untranslated sequence of the cDNA. This 3' non-translated sequence contains six in-phase termination codons. Exons 2-4 correspond to the rest of the coding sequence.

We have used the human cDNA fragment for Southern and Zoo-blot analysis (data not shown). The Southern blots of human DNA show a single hybridising band with several different restriction endonucleases suggesting that this gene does not belong to a complex gene family. This sequence is highly conserved among species as we have detected strong signals with bovine, mouse, chicken, dog, porcine, sheep and rat genomic DNAs.

Discussion

We have used RACE-PCR with nested amplimers to isolate the 5' and 3' ends of the human NK-2 receptor, and this is the first report of a complete cDNA being isolated by this method. Analysis of RNA expression levels has shown that this receptor is of low abundance in human lung and this is confirmed by the PCR work where two rounds of amplification were required in order to detect product.

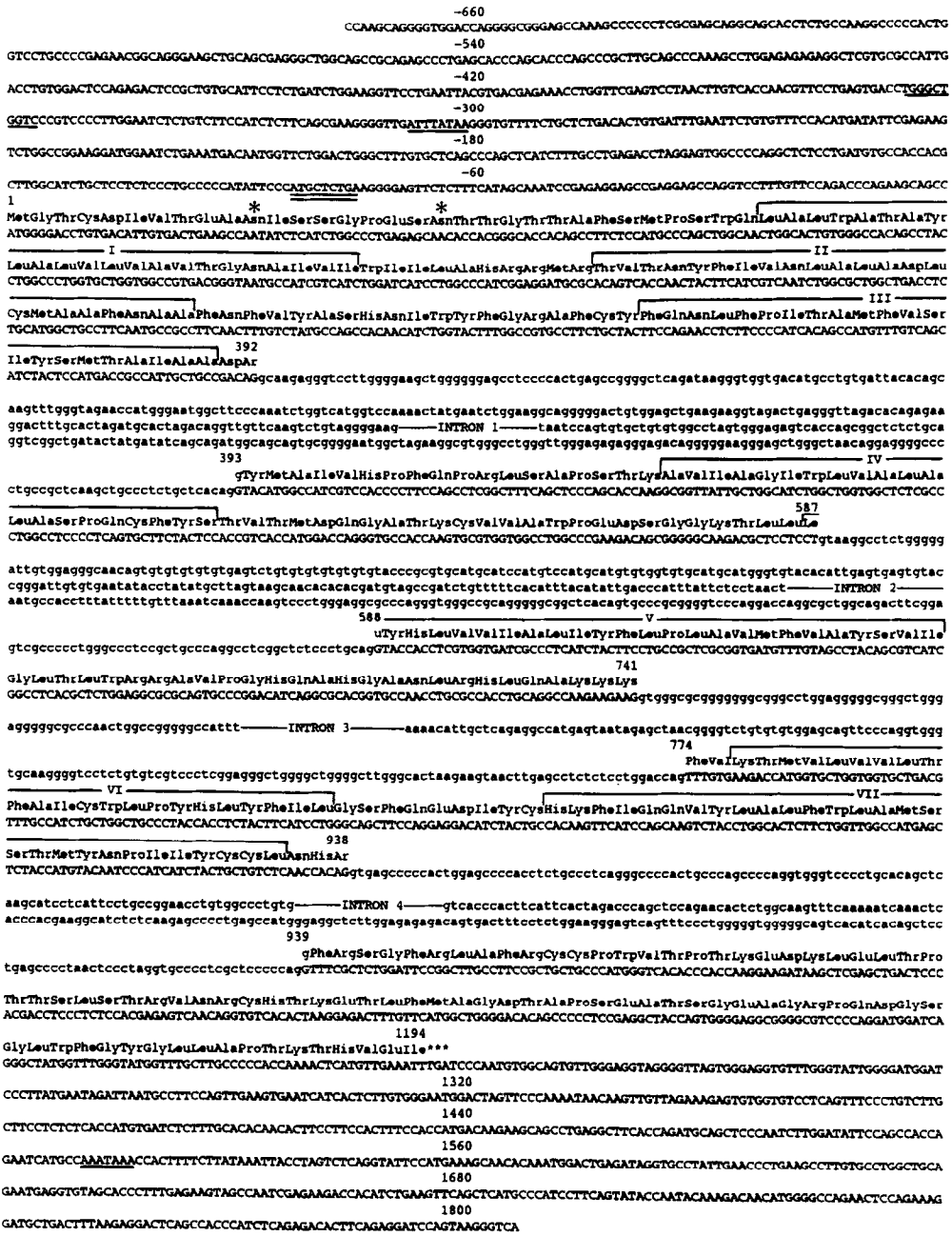

Fig.4. Nucleotide sequence of the human NK-2 receptor gene. The DNA sequence of the human NK-2 receptor gene and the deduced amino acid sequence, beginning with the initiator methionine, is shown. The nucleotide residues are numbered with respect to the cDNA sequence with the A of the initiator methionine numbered as 1, and nucleotides on the 5' side of nucleotide 1 being indicated by negative numbers. Sequences similar to the TATA box (ATTTATAA) and GC box are shown underlined as is the presumed polyadenylation signal. Postulated N-glycosylation sites are indicated by asterisks. Positions of the putative transmembrane segments I-VII of NK-2R are indicated above the amino acid sequence. Double underline, a small open reading frame in the 5' non-coding region. The 5' and 3' limits of the nucleotide sequences obtained by the RACE-PCR method are shown by arrows.

Recently the human NK-2 receptor has been isolated from trachea (24) and from jejunum (25) by standard hybridisation methods. The protein coding sequences are identical except at His^{241} where Gerard et al report Leu^{241} (24), and at the two polymorphic sites mentioned above. These authors also have seventeen differences in sequence in the 3' untranslated region, one in the 5' untranslated region and several in the intronic sequences compared to the sequence reported here. The differences in the 5' and 3' untranslated do not appear to be due to polymorphisms as we have sequenced these regions from several individuals and they are all identical.

Cloning of the gene for a human NK-2 receptor will facilitate investigation of the molecular processes involved in activation and regulation of this receptor, the potential role of these receptors in airway function and to plan more effective treatment for lung disorders.

Acknowledgments

We would like to thank Philip Hedge for the human cosmid library. We are grateful to Dave Holland and Richard Hughes for oligonucleotide synthesis, to David Black for help with microinjections, and to Nick Sturgess for useful discussions.

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